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Priming of STAT1 and STAT3 for Cytokine-triggered Degradation by the Proteasome upon A_{2A}Adenosine Receptor (A_{2A}AR) Expression

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Acknowledgements **ii**
Abbreviations **iii**
Table of Contents **viii**
List of Figures **xi**
List of Table **xv**
Abstract **xvi**

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Abbreviations

A_{2A}AR	A_{2A} adenosine receptor
ADP	Adenosine 5´-diphosphate
AMP	Adenosine 5´-monophosphate
APR	Acute phase response
AR	Adenosine receptors
ARHI	A Ras homologue member I
ATP	Adenosine triphosphate
AVs	Adenoviruses
BCA	Bicinchonic acid salt
BMI	Body mass index
BSA	Bovine serum albumin
BSF-3	B-cell-stimulating factor-3
CaM	Calmodulin
cAMP	Cyclic AMP
CBM	Cytokine-binding module
C-ERMAD	C-terminal ERM-associated domain
cGMP	Cyclic GMP
CIS	Cytokine-inducible SH2 protein
CNTF	Ciliary neurotrophic factor
CRP	C-reactive protein
CT-1	Cardiotrophin-1
CUE	Coupling of Ub conjugation to ER degradation
Cul4A	Cullin 4A
DMAM	Dulbeco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
dNTPs	Deoxynucleotide 5´-triphosphates
DTT	Dithiothreitol
DUB	Deubiquitylating enzyme
EBM-2	Endothelial Basal Media-2™
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ECs	Endothelial cells
EDTA	Ethylenediaminotetra-acetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetracetic acid
EPAC	Exchange protein activated by cAMP
EPO	Erythropoietin
ERK	Extracellular-signal regulated kinase
ERM	Ezrin-radixin-moesin
FERM	Four-point-one ezrin radixin moesin

FBS	Foetal bovine serum
FBS	Phosphate-buffered saline
GAGs	Glycosaminoglycans
G-CSFR	Granulocyte colony-stimulating factor receptor
GH	Growth hormone
GM	Granulocyte-macrophages
Gp 130	Glycoprotein 130
HAEC	Human aortic endothelial cells
HEPES	N-(2-Hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)
hFGF-B	Human fibroblast growth factor-B
HRP	Horseradish peroxidase
IFN	Interferon
IGF-l	Insulin-like growth factor-l
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IKK	IκB kinase
IL	Interleukin
IL-6Rα	IL-6 receptor α
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAK	IL-1 receptor-associated kinase
JAK	Janus kinase
JH	JAK homology
KIR	kinase inhibitory region
LIF	leukaemia inhibitory factor
LIF-R	leukemia inhibitory factor receptor
LPS	lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MD-2	Myeloid differentiation protein-2
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MI	Myocardial infarction
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl 2H-tetrazolium bromide
MTX	Methotrexate
NCA	Neutrophil cytoplasmic antibody
NCAM	Neural cell adhesion molecule
NEDD8	Neural precursor cell expressed developmentally down-regulated 8
NF-κB	Nuclear factor-κB
NK	Natural killer
NLS	Nuclear localisation sequence
NNT-1	Neurotrophin-1
NPC	Nuclear pore complex
NRTK	Non-receptor tyrosine kinase
OSM	Oncostatin M
PH	Pleckstrin homology

PIAS	Protein inhibitor of activated STAT
PKA	Protein kinase A
PlGF	Placental growth factor
PLR	Prolactin
PMN	Polymorphonuclear
PMSF	Phenylmethylsulphonyl fluoride
PTK	Protein tyrosine kinase
PTPase	Protein tyrosine phosphatase
RANTES	Regulated upon activation normal T cell expressed presumed secreted
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAA	Serum amyloid
SCID	Severe combined immunodeficiency disease
SDS	Sodium dodecyl sulphate
SH2	Src homology2
SHP2	SH2-domain-containing protein tyrosine phosphatase
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
StIP1	STAT3 interacting protein
SUMO	Small ubiquitin-like modifier
TAP	Two-step tandem affinity purification
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	Transforming growth factor-β
Th1	Type-1 T-helper cells
TMB	3,3'5,5' –tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
TPO	Thrombopoietin
TRIS	Tris(hydromethyl-amino)ethane
TSLP	Thymic stromal lymphopoietin
Ub	Ubiquitin
UBA	Ub-associated domain
UBD	Ub-binding domain
UBL	Ub-like domain
UBP	Ub-specific processing protease
UIM	Ub-interacting motif
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VIPs	V-interaction proteins
WT	Wild-type

Pharmacological Names

CGS21680 2-*p*-(2-Carboxyethyl)phenethylamino-5´-N-ethylcarboxamidoadenosine hydrochloride

ZM241385 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-α}{1,3,5}triazin-5-yl-amino]ethyl)phenol

Table of Contents

Chapter 1 Introduction 1

1.1 JAKs in cytokine signalling 2
1.2 The Signal transducer and activator of transcription (STAT) family of transcription factors 4
1.3 Regulation of cytokine signalling by suppressor of cytokine signalling (SOCS) family molecules 8
1.3.1 SOCS1 9
1.3.2 SOCS3 10
1.4 Cytokines 12
1.4.1 IL-6 13
1.4.1.1 IL-6 structure 14
1.4.1.2 IL-6 Receptors 14
1.4.1.3 Signalling of IL-6-type cytokines 17
1.4.2 IFN alpha 18
1.4.2.1 IFN-inducible STAT complexes 20
1.4.3 Leptin Biology 23
1.4.3.1 Leptin receptor 24
1.5 Adenosine receptors 25
1.5.1 A_{2A}AR 26
1.5.1.1 The A_{2A}AR as anti-inflammatory GPCR 29
1.6 Ubiquitination 33
1.6.1 Ub as an inducible and reversible signal 34
1.6.2 Proteasomal degradation 35
1.7 E3 ligase and STAT degradation 36
1.8 Aims and objectives of research 38

Chapter 2 Materials and Methods 41

2.1 Chemicals and Suppliers 42
2.2 Cell Culture & Transfection Methods 46
2.2.1 Cell maintenance 46
2.2.2 Transfection with Fugene 47
2.2.3 Infection of HUVECs with AVs 47
2.3 Molecular Biology 47
2.3.1 Preparation of antibiotic agar plates 47
2.3.2 Preparation of competent BL21 *E.coli* 48
2.3.3 Transformation of competent BL21 *E.coli* 48
2.3.4 Preparation of plasmid DNA 48
2.3.5 Digestion of plasmid DNA 49
2.3.6 Preparation of RNA 49
2.3.7 Reverse transcription-PCR (RT-PCR) 50
2.3.8 PCR of prepared cDNA 50
2.3.9 Purification of adenoviral vectors 51
2.3.10 Preparation of GST-UBA Sepharose beads 53

2.3.11 SPAGE assays for quantitation of immobilised GST fusion proteins. 53
2.4 Laboratory techniques....56
2.4.1 Cell lysis....56
2.4.2 Discontinuous SDS-PAGE and Immunoblotting....56
2.4.3 Protein concentration determination using the bicinchonic acid (BCA) protein assay....57
2.4.4 GST-Dsk2pUBA pull-down assays....60
2.4.5 Immunoprecipitation....61
2.4.6 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl 2H-tetrazolium bromide (MTT) assay of cell viability....63
2.4.7 Statistical Analysis....63

Chapter 3 Effect of A_{2A}AR Expression on Cytokine Activation of the JAK-STAT pathway in vascular ECs....64

3.1 Introduction....65
3.2 Results....67
3.3 Discussion....98

Chapter 4 Mechanisms Controlling the Effects of A2AAR Expression on Cytokine-Stimulated Degradation of STATs....104

4.1 Introduction....105
4.2 Results....107
4.3 Discussion....131

Chapter 5 Effect of A2AAR Expression on STAT3 Regulated Genes....138

5.1 Introduction....139
5.2 Results....141
5.3 Discussion....152

Chapter 6 Final Discussion....159

Chapter 7 Future Experimental Approaches……167

Chapter 8 Bibliography....175

List of Figures

Chapter 1

Figure 1.1 Structure organisations of JAKs, STAT factors, Tyrosine Phosphatase (SHP2) and SOCS proteins. 4
Figure 1.2 SOCS family members might target signalling proteins for degradation by the proteasome. 9
Figure 1.3 Receptor complexes of IL-6-type cytokines. 15
Figure1.4 Schematic presentation of gp130 mediated activation of JAK/STAT pathway and MAPK / ERK cascade by IL-6 signalling 18
Figure 1. 5 Primary JAK/STAT pathways involved in cellular signalling by type I and type II IFNs. 21
Figure 1. 6 Overview of the Ub Conjugation Pathway 36
Figure 1.7 Schematic model of JAK/STAT regulation through the ubiquitin-proteasome pathway. 38

Chapter 2

Figure 2.1 SDS-PAGE protein assay for immobilised GST fusion proteins on beads 55

Chapter 3

Figure 3.1 A_{2A}AR gene transfer to HUVECs 71
Figure 3.2 Effect of A_{2A}AR gene transfer on STAT1 phosphorylation and expression in response to sIL6Rα/IL-6 73
Figure 3.3 Effect of A_{2A}AR gene transfer on STAT3 phosphorylation and expression in response to sIL6Rα/IL-6 75
Figure 3.4 Effect of A_{2A}AR gene transfer on STAT1 phosphorylation and expression in response to IFNα 77
Figure 3.5 Effect of A_{2A}AR gene transfer on STAT3 phosphorylation and expression in response to IFNα 79
Figure 3.6 Effect of A_{2A}AR gene transfer on STAT1 expression in response to prolonged exposures to IL-6Rα/IL-6 81

Figure 3.7 Effect of A_{2A}AR gene transfer on STAT3 expression in response to prolonged exposure to sIL-6Rα/IL-6 82
Figure 3.8 Effect of A_{2A}AR gene transfer on STAT1 expression in response to prolonged exposure to IFNα 83
Figure 3.9 Effect of A_{2A}AR gene transfer on STAT3 expression in response to prolonged exposure to IFNα 84
Figure 3.10 Effect of A_{2A}AR on STAT1 phosphorylation and expression in response to sIL-6 Rα/ IL-6 85
Figure 3.11 Effect of A_{2A}AR on STAT3 phosphorylation and expression in response to sIL-6 Rα/ IL-6 87
Figure 3.12 Effect of A_{2A}AR expression and sIL-6Rα/IL-6 expression on HUVECs viability 89
Figure 3.13 Effect of MG132 on sIL-6Rα/IL-6-induced STAT1 downregulation in A_{2A}AR-expressing HUVECs 90
Figure 3.14 Effect of MG132 on sIL-6Rα/IL-6-induced STAT3 downregulation in A_{2A}AR-expressing HUVECs 91
Figure 3.15 Effect of MG132 on IFNα-induced STAT1 downregulation in A_{2A}AR-expressing HUVECs 92
Figure 3.16 Effect of MG132 on IFNα-induced STAT3 downregulation in A_{2A}AR-expressing HUVECs 93
Figure 3.17 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT1 phosphorylation in response to sIL-6 Rα/IL-6 94
Figure 3.18 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT3 phosphorylation in response to sIL-6 Rα/IL-6 95
Figure 3.19 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT1 phosphorylation in response to IFNα 96
Figure 3.20 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT3 phosphorylation in response to IFNα 97

Chapter 4

Figure 4.1 Effect of JAK inhibition 1 on sIL-6Rα/IL-6-induced JAK1, JAK2 and STAT1 phosphorylation 112

Figure 4.2 Effect of JAK inhibition on sIL-6Rα/IL-6-induced STAT1 phosphorylation and downregulation of STAT1 in A_{2A}AR expressing HUVECs 114
Figure 4. 3 Effect of JAK inhibition on sIL-6Rα/IL-6-induced STAT3 downregulation in A_{2A}AR expressing HUVECs 115
Figure 4.4 Effect of JAK inhibition on IFNα-induced STAT1 downregulation in A_{2A}AR expressing HUVECs 116
Figure 4.5 Effect of JAK inhibition on IFNα-induced STAT3 downregulation in A_{2A}AR expressing HUVECs 117
Figure 4.6 Leptin stimulates the specific phosphorylation of STAT3 but not STAT1 118
Figure 4.7 Effect of A_{2A}AR gene transfer on STAT3 phosphorylation and expression in response to leptin 119
Figure 4.8 Effect of A_{2A}AR gene transfer on STAT1 and STAT3 expression in response to prolonged exposure to leptin..... 121
Figure 4.9 Effect A_{2A}AR gene transfer on WT and Tyr705→Phe mutated STAT3 expression levels. 123
Figure 4.10 Effect of A_{2A}AR gene transfer on mRNA levels of the phospho STAT-specific E3 Ub ligase SLIM/Mystique in vascular endothelial cells under conditions that promote STAT degradation 124
Figure 4.11 Effect of A_{2A}AR gene transfer on protein expression of the phospho STAT-specific E3 Ub ligase SLIM/Mystique in vascular endothelial cells under conditions that promote STAT degradation..... 125
Figure 4.12 Effect of A_{2A}AR gene expression on SOCS3 induction in response to sIL-6 Rα/IL-6..... 126
Figure 4.13 A_{2A}AR expression primes STAT3 for cytokine-triggered ubiquitylation 127
Figure 4.14 A_{2A}AR expression primes STAT1 for cytokine-triggered ubiquitylation 128
Figure 4.15 Effect of UBP5 on release of STAT1 from GST-Dsk2p..... 129
Figure 4.16 A_{2A}AR expression primes Flag-WT but not Tyr705→Phe mutated STAT3 for cytokine-triggered ubiquitylation..... 130

Chapter 5

Figure 5.1 Effect of sIL-6Rα/IL-6 on VEGFR2 expression 144
Figure 5.2 Effect of JAK inhibition on sIL-6Rα/IL-6-induced VEGFR2 expression in HUVECs 145
Figure 5.3 Effect of WT and Tyr705→Phe mutated STAT3 expression on VEGFR2 expression in HUVECs 146
Figure 5.4 Effect of A_{2A}AR gene transfer on VEGFR2 expression in response to sIL-6Rα/IL-6..... 148
Figure 5.5 Effect of WT and Tyr705→Phe mutated STAT3 expression on eNOS expression 149
Figure 5.6 Effect of sIL-6Rα/IL-6 on eNOS expression 150
Figure 5.7 Effect of A_{2A}AR gene transfer on eNOS expression in response to sIL-6Rα/IL-6..... 151

List of Table

Chapter 2

Table 2.1 Antibody Incubation Conditions……58

Abstract

The A_{2A} adenosine receptor (A_{2A}AR) functions as a key non-redundant suppressor of inflammatory responses *in vivo*. However, whether it regulates activation of the JAK-STAT pathway utilised by many pro-inflammatory cytokines is unknown. Using a vascular endothelial cell model system, I have demonstrated that adenovirus-mediated expression of the human A_{2A}AR conferred an ability of IFNα, leptin and a soluble IL-6 receptor-α/IL-6 (sIL-6Rα/IL-6) trans-signalling complex to promote a time-dependent reduction in the levels of STAT proteins that was entirely due to proteasomal degradation. In terms of functional consequences, degradation was sufficient to attenuate sIL-6Rα/IL-6-stimulated STAT3-dependent up-regulation of vascular endothelial growth factor receptor-2 (VEGFR-2) and enhance eNOS expression. Degradation required JAK activity since A) it was blocked by preincubation with JAK inhibitor. B) STAT1 but not STAT3 was resistant to both tyrosine phosphorylation and down-regulation in response to leptin and C) a Tyr705→Phe mutated STAT3 was also resistant to cytokine-triggered degradation, suggesting that JAK-mediated phosphorylation of this residue is required to produce the effect. Consistent with this hypothesis, sIL-6Rα/IL-6 treatment of A_{2A}AR-expressing cells resulted in the accumulation of polyubiquitylated endogenous and epitope-tagged recombinant wild-type but not Tyr705→ Phe-mutated STAT3. In addition the results show that inhibition of proteasome function was sufficient to block the inhibitory effect of the A_{2A}AR on STAT3 phosphorylation, demonstrating that priming of STATs for degradation is the only mechanism responsible for the reduced cytokine-stimulated STAT phosphorylation observed in A_{2A}AR-expressing cells. To date there is only one E3 ligase known for mediating STAT degradation which is SLIM protein. However, our results suggest the involvement of another E3 ubiquitin ligase in HUVECs, since we have been unable to detect SLIM message or protein in HUVECs under conditions in which STAT degradation occurs. In addition, while Tyr- phosphorylation is clearly the critical step in targeting STATs for degradation in A_{2A}AR-expressing cells, it is unclear as to whether it functions simply as a classical phosphodegron, or whether the nuclear translocation that occurs as a result of phosphorylation is also important for localising the phosphorylated STAT dimer with the relevant E3 ubiquitin ligase.

Together, these observations suggest a model whereby expression of the A_{2A}AR in endothelial cells primes JAK-phosphorylated STATs for polyubiquitylation and subsequent degradation by the proteasome following cytokine treatment, and represents a previously unappreciated mechanism by which G-protein-coupled receptors can negatively regulate responsiveness to specific JAK-STAT-mobilising adipocytokines acting on the vascular endothelium.

Chapter1

Introduction

1.1 JAKs in cytokine signalling

JAKs are intracellular tyrosine kinases with molecular masses of 120-140 KDa. It was demonstrated that signalling of IL-6 crucially depends on the presence of JAK1. The interaction between gp130 and JAK1 is very tight and long lasting. JAKs bind to the membrane-proximal region of IL-6 receptors containing box1 and box2 motifs. The general structure of JAKs is shown in (Figure1.1) (Murakami *et al.*; 1993; Haan *et al.*; 2006).

Comparison of JAKs sequences reveals seven regions of high homology from JH1 to JH7. The C-terminal JH1 tyrosine kinase domain is preceded by a JH2 pseudokinase domain, which is devoided of catalytic activity, but regulates the activity of the kinase domain (Yamaoka *et al.*; 2004). Moreover, JAKs contain an SH2 domain. The N-terminal region of JAKs comprises a FERM domain, which is important for receptor association (Haan *et al.*; 2006; Haan *et al.*; 2008) (Figure 1.1).

JAKs are constitutively associated with the proline-rich membrane proximal box1/box2 region. Upon ligand stimulation, receptors undergo conformational changes that bring JAKs into close proximity to each other, enabling activation by trans-phosphorylation (Haan *et al.*; 2006). There are four members of the JAK family in mammals; JAK1 plays a critical role in mediation of biological responses to several major cytokine receptor families, while JAK2 plays a critical role in transducting signals for Epo, IL-3, GM-SF, IL-5, Tpo and IFN-γ. JAK3 plays a vital role in lymphoid development (Giliani *et al.*; 2005) while Tyk2 appears to be most important in mediating the biological response to IL-12 and LPS. JAK1, JAK2 and Tyk2 are expressed ubiquitously, whereas the expression of JAK3 is restricted to cells of the myeloid and lymphoid lineages (Cetkovic-Cvrlje and Uckun; 2004).

The importance of JAKs in mediating signals from a variety of cytokines factors underscores their importance in signal transduction in general. However, the complexity associated with processing signals from such diverse sources suggests a

complicated mechanism of action for the JAK kinases. JAK activation is determined by an in vitro kinase assay that measures an increase in tyrosine phosphorylation of substrates. JAKs, when expressed in the baculovirus system are enzymatically active and are phosphorylated on tyrosine residues (Dezaire and Stark; 2007). Their overexpression in mammalian cells also leads to constitutive activation, most probably due to dimerisation (Staerk *et al.*; 2007). On the other hand, a JAK kinase in complex with a native un-liganded receptor is in a catalytically inactive latent state. Receptor dimerisation/oligomerisation due to ligand binding results in the juxtapositioning of the JAKs, which are in the vicinity either through homo- or hetero-dimeric interactions. This recruitment of the JAK kinases appears to result in either via autophosphorylation and/or cross phosphorylation by other JAK kinases or other tyrosine kinase family members. This activation is presumed to result in an increased JAK kinase activity. The phosphotyrosine sites on the receptors can then serve as docking sites that allow the binding of other SH2-domain containing signalling molecules such as STATs, Src-kinases, protein phosphatases and other adaptor signalling proteins such as Grb2 (Heinrich *et al.*; 2003).

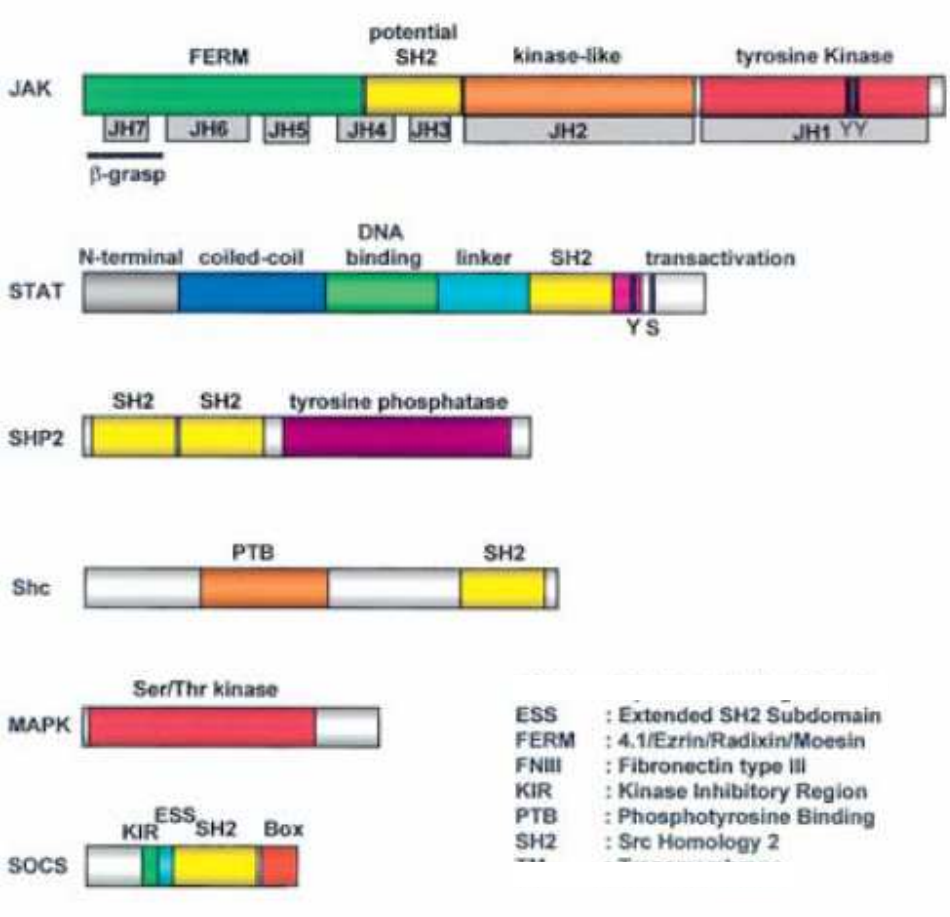


Figure 1.1 Structure organisations of JAKs, STAT factors, Tyrosine Phosphatase (SHP2) and SOCS proteins.

The JAK kinases are divided into seven JAK homology (JH) domains starting from the tyrosine kinase domain located at the C-terminus of JAKs and designated as JH1. The tandem kinase domain structure is composed of the tyrosine kinase domain, JH1, and a pseudokinase or kinase-like domain, JH2, on the N-terminal side of JH1. Only the JH1 domain is catalytically active (Heinrich *et al.*; 2003).

1.2 The Signal transducer and activator of transcription (STAT) family of transcription factors

The mammalian STAT factors are designated as STAT1, 2, 3, 4, 5a, 5b and 6. STATs are proteins with a conserved structural organisation (Figure1.1). They consist of 750-850 amino acids and comprise various domains as follows: a tetra-

merisation domain and a leucine-zipper-like domain at the N-terminus, DNA-binding domain in the middle, a Src homology 3 (SH3)-like domain, a SH2/tyrosine activation domain, linker domain, and a transactivation domain at the C-terminus. The C-terminal transcriptional activation domain is quite divergent between STAT members and contributes to signalling specificity (Lim and Cao; 2006).

The N-terminal domain comprising approximately 130 amino acids is conserved among the STATs. Several studies suggest that the N-terminal dimerisation promotes cooperativity of binding to tandem GAS elements. Other studies have suggested that the terminal STAT domain promotes interaction with the transcriptional coactivator CBP and p300, PIAS family members, receptor domains, as well as regulating nuclear translocation (Horvath; 2000; Lim and Cao; 2006).

The coiled-coil domain consists of four α-helices. The crystal structures of STAT1 and STAT3 reveal that this domain forms a large predominantly hydrophilic surface that is available for specific interaction with other helical proteins. Proteins interacting with the coiled-coil domain include IFN regulatory factor-9 (IRF-9), the transcription factor c-jun, N-myc interactor (Nmi) and STAT3 interacting protein (StIP1). In addition the coiled-coil domain is also implicated in receptor binding, tyrosine phosphorylation and nuclear export (Kisseleva *et al.*; 2002; Lim and Cao; 2006).

Most investigators have found an increase in transcription of target genes after cytokine-induced serine phosphorylation of STAT1, STAT3 and STAT5. Earlier studies had already shown serine^{727} phosphorylation of STAT3 to occur slower than the phosphorylation of Tyr^{705} (Beuvink *et al.*; 2000). SH2 domains play an important role in signalling through their ability to bind to specific phosphotyrosine motifs. Activation of STATs requires phosphorylation of the conserved tyrosine residue located directly on the C-terminal side of the SH2 domain (Tyr^{705} in STAT3). During this process, the STATs are recruited to tyrosine phosphorylated cytokine receptors

through their SH2 domains, and following phosphorylation of the conserved tyrosine, the STATs undergo dimerisation through a reciprocal SH2-phosphotyrosine interaction (Greenlund *et al.*; 1994; Mayya and Loew; 2005). Only the STAT dimer is transcriptionally active, and STAT monomers are unable to bind DNA, and importantly, only the dimer translocates to nucleus from the cytoplasm, where the STATs are activated. Furthermore, during activation of STATs, the SH2 domain has been found to be required for interaction with and subsequent phosphorylation by JAKs (Gupta *et al.*; 1996). Mutation of either the conserved phosphotyrosine-binding Arg of the SH2 domain or the conserved tyrosine following the SH2 domain abrogates STAT activity (Kim *et al.*; 1998). Differences in the SH2 domains of STATs determine, at least partially, the specificity of STAT binding to various cytokine receptors (Heim *et al.*; 1995; Zhukovskaya *et al.*; 2004).

Although the sequence of the STAT SH2 domain is quite divergent from other SH2 domains, its tertiary-structure is well conserved (Gao *et al.*; 2004). It consists of an anti parallel β-sheet flanked by two α-helices, which form a pocket. An absolutely conserved arginine, which mediates the interaction with phosphate, lies at the base of this pocket (Arg-602 for STAT1 and Arg-609 for STAT3). The ability of the SH2 domain to recognise specific phosphotyrosine motifs play an essential role in three STAT signalling events; recruitment to the cytokine receptor through recognition of specific phosphotyrosine motifs, association with the activating JAK STAT homodimerisation or heterodimerisation, STAT dimerisation depends on the interaction between the SH2 domain of one STAT monomer and the tyrosine phosphorylated tail segment of the other monomer (T. Kawata; 1997; Mayya and Loew; 2005).

STAT dimerisation is a pre-requisite for DNA binding. The DNA-binding domain of STAT proteins is located in the middle of the molecule (amino acid 300-480). STATs are activated in the cytoplasm; however they function within the nucleus (Matsukawa; 2007). In general, serine phosphorylation of STAT1, STAT2, STAT3

and STAT4 increase their signal potential (Chung *et al.*; 1997b; Schuringa *et al.*; 2000).

STAT dimers need to cross the nuclear envelope to functionally link the cell membrane with the promoters of cytokine-responsive genes. Movement of STATs in either compartment is diffusion controlled and not directed along permeant structures. Passage through nuclear gateways (nuclear pore complexes NPCs) (Rabut *et al.*; 2004). NPCs form channels in the nuclear envelope with a diameter of ~ 40 nm (Laskey; 1998; Vasu and Forbes; 2001). Vertebrate NPCs have a mass of ~125 MDa and contain 30–50 different proteins, which are called nucleoporins. Small molecules of 40 kDa can passively diffuse through the NPC (Caesar *et al.*; 2006). In contrast, the translocation of larger macromolecules into the nucleus occurs via an active mechanism involving nuclear transport receptors. The majority of the nuclear transport pathways are mediated by receptors of the importin family. Proteins or other cargo molecules that carry a classical nuclear localisation sequence (NLS) are recognised by importin-α, which subsequently forms a complex through its importin-β-binding domain with importin-β (Gorlich *et al.*; 1996; Caesar *et al.*; 2006). Analysis of their nucleo-cytoplasmic translocation has to consider that the STAT proteins exist in two different states in terms of signalling: Before the stimulation of cells with cytokines the STAT molecule exists in a nontyrosine phosphorylated state, the oligomerisation status of which is still debated (Hurt; 1997; Meyer and Vinkemeier; 2004).

Reporter gene studies have determined that serine phosphorylation enhances transcriptional activity of STAT1 and STAT3 (Kovarik *et al.*; 2001). More physiological studies in STAT1-deficient cells reconstituted with STAT1 and $STAT1^{S727A}$ demonstrated that serine phosphorylation only enhances the ability of STAT1 to drive expression of some, but not all target genes (Kovarik *et al.*; 2001; Shi and Kehrl; 2004). Many studies also indicate that serine phosphorylation enhance STAT4 transcriptional activity. Although both STAT5 and STAT6 can become serine

phosphorylated, enhanced transcriptional activity has not been convincingly demonstrated (Kovarik *et al.*; 2001).

1.3 Regulation of cytokine signalling by suppressor of cytokine signalling (SOCS) family molecules

Cytokine signalling is negatively regulated in part by suppressor of cytokine signalling (SOCS), which bind to tyrosine phosphorylated residues of target proteins via their SH2 domains (Krebs and Hilton; 2001; Johnston; 2004). They inhibit JAK activity through their N-terminal domains and are thought to induce degradation of bound molecules through a conserved SOCS box motif that interacts form part of an E3 ubiquitin (Ub) ligase complex (Zhang *et al.*; 2001).

SOCS proteins are characterised by a relatively divergent N-terminal region followed by a SH2 domain and a C-terminal SOCS box region. SOCS proteins inhibit IL-6 signalling either by suppressing the kinase activity of JAKs or by direct interaction with the receptors or by both mechanisms (Yasukawa *et al.*; 1999; Kurdi and Booz; 2007).

It is becaming increasingly appreciated that SOCS proteins also act by promoting the degradation of specific signalling proteins. Recent work suggests that SOCS box-containing proteins act as adapter molecules that recruit activated signalling proteins to the proteasome. SOCS proteins interact with elongins B and C through their SOCS box. In turn, elongin BC complexes associate with cullin 2 and a ring finger-containing protein Rbx1 (Roc1) to form part of a putative E3 Ub ligase (Figure1.2). The SH2 domains of SOCS bind to tyrosine phosphorylated signalling proteins. They can therefore act as adapters to facilitate the polyubiquitination and subsequent degradation of associated signalling proteins (Zhang *et al.*; 1999a; Johnston; 2004).

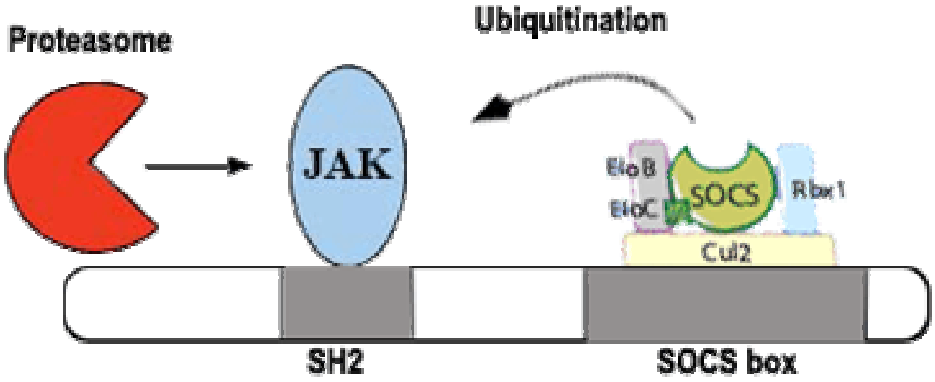


Figure 1.2 SOCS family members might target signalling proteins for degradation by the proteasome.

All SOCS proteins bind the elongin BC complex through their SOCS box. In turn, the elongin BC complexes associate with cullin 2 and a ring finger-containing protein Rbx1 (Roc1) to form part of a putative E3 Ub ligase. Signalling proteins associated with the N-terminal or SH2 domains of SOCS proteins could be ubiquitinated by cullin-2 targeting them for degradation by the proteasome.

1.3.1 SOCS1

SOCS1 was initially reported as a molecule induced by STATs. However, SOCS1 expression is now known to be induced by insulin (Le *et al.*; 2002; Ueki *et al.*; 2004), LPS (Crespo *et al.*; 2000; Mostecki *et al.*; 2005), CpG DNA and other molecules that do not use STATs in signal transduction. The SH2 domain of SOCS1 recognises the phosphorylated tyrosine residue located in the activation loop of the JAK kinase domain (Brysha *et al.*; 2001; Greenhalgh and Hilton; 2001). However, inhibition of JAK activation requires not only the SH2 domain but also a 30 amino acid kinase inhibitory region located upstream of the SH2 domain (Waiboci *et al.*; 2007).

Many studies suggest that SOCS1 inhibits cytokine signalling by associating with JAK1, JAK2, JAK3 and TYK2 to inhibit their catalytic activity. Structure-function studies using truncated or chimeric version of SOCS1 have revealed the mechanism by which SOCS proteins bind to and inhibit JAKs. Interestingly, although the SOCS1 SH2 domain is sufficient to mediate the association between SOCS1 and JAKs (Yasukawa *et al.*; 2000), both the SH2 domain and 24 residues immediately N-terminal to the SH2 domain is necessary for inhibition of JAK2 activity. Thus, the region immediately N-terminal to the SOCS1 SH2 domain appears to have a kinase inhibitory function (Waiboci *et al.*; 2007). Within this N-terminal region SOCS1 contains a sequence of 12 residues that resembles the JAK activation loop (Yasukawa *et al.*; 1999). In vivo study of SOCS function has been facilitated by SOCS1 knockout mice (Alexander *et al.*; 1999). Phenotypically, $SOCS1^{-/-}$ mice are stunted and die at three weeks of age. These mice have the pathology characterised by severe lymphopenia, fatty degeneration of the liver and macrophage infiltration of major organs (Starr *et al.*; 1998). The complex disease in $SOCS1^{-/-}$ mice was prevented by administration of anti-IFN-γ antibodies and did not occur in $SOCS1^{-/-}$ mice also lacking the IFN-γ gene (Fenner *et al.*; 2006). Subsequent studies have established that mice lacking SOCS1 show both increased production of and heightened sensitivity to IFN-γ which contribute to the perinatal lethality of SOCS1 deletion (Brysha *et al.*; 2001).

1.3.2 SOCS3

The structure of SOCS3 is similar to SOCS1, including the position of a kinase inhibitory region (KIR). However, these two molecules differ greatly in their mechanism of action; SOCS1 can inhibit activation of JAK by directly binding to JAK, while SOCS3 inhibit the action of JAK only in the presence of receptors, such as gp130. SOCS3 is induced by IL-6 and SHP2 are simultaneously activated (Terstegen *et al.*; 2000; Kisseleva *et al.*; 2002; Lehmann *et al.*; 2003) .

It has also been observed that an inhibition of SHP2 activation can lead to an enhanced induction of SOCS3 mRNA. On the other hand the expression of the SOCS3 protein decreased the level of tyrosine-phosphorylated SHP2 after IL-6 stimulation. Furthermore, SOCS3 inhibits IL-6 signalling by inhibiting the phosphorylation of gp130. SOCS3 has been found to bind to the phosphotyrosine motif 759 of gp130 which is also the binding site for SHP2 (Kisseleva *et al.*; 2002). The affinity of SOCS3 to a phosphotyrosine peptide corresponding to the Tyr^{759} motif of gp130 is much higher than to a phospho-peptide comprising the JAK activation loop. Moreover, the affinity of SOCS3 to bind gp130 is slightly higher than that of SHP2. Thus the involvement of SHP2 and SOCS3 in Tyr^{759} mediated attenuation has been re-examined to determine the individual contribution of both proteins (Yasukawa *et al.*; 1999). Although both SOCS3 and SHP2 are recruited to the same site within gp130, there are two largely distinct modes of negative regulation of gp 130 activity: through the feedback inhibition by SOCS3 or the dephosphorylation of phosphorylated JAKs, receptors and STATs by SHP-2 (Lehmann *et al.*; 2003). The proposed mechanisms of action of SOCS 1 and SOCS3 have indicated that the N-terminal domains of each protein (but not the N-terminal domain of any other SOCS protein) are interchangeable. This suggests that SOCS1 and SOCS3 may inhibit JAKs through interaction with their N-terminal domains (Yasukawa *et al.*; 2000; Krebs and Hilton; 2001). It has been shown by several investigators that SOCS1 and SOCS3 bind to the kinase domain of activated JAK1 and JAK2 (Marine *et al.*; 1999; Sasaki *et al.*; 1999; Park *et al.*; 2003). A model for JAK inhibition by SOCS1 suggests that the kinase activation loop of JAK2 interacts with the SH2 domain of SOCS1. This allows SOCS to present its KIR, which is quite homologous to the kinase activation loop, to the pocket in the activation site, which in turn might prevent the access of substrates or ATP (Alexander and Hilton; 2004).

The physiological function of SOCS3 has been investigated. *In vivo* expression pattern of SOCS3 revealed that although SOCS3 is expressed at low levels in adult tissues, it is highly expressed in fetal liver erythroid progenitors (Marine *et al.*; 1999).

Furthermore, SOCS3 expression is specifically induced during a stage of erythropoiesis characterised by an explosive Epo-dependent expansion of cells of the erythroid lineage, leading to the hypothesised that SOCS3 plays a role in erythropoiesis (Sasaki *et al.*; 2000). It is interesting to note that neither the $SOCS3^{-/-}$ mice nor the transgenic mice that overexpress SOCS3, survived to birth. Strikingly, transgenic embryos that constitutively overexpressed SOCS3 exhibited no detectable fetal liver erythropoiesis, whereas a proportion of $SOCS3^{-/-}$ embryos died, exhibiting pathology characterised by massive erythrocytosis throughout the embryo (Marine *et al.*; 1999) and defects in the placenta (Roberts *et al.*; 2001). lethality in $SOCS3^{-/-}$ mice results from placental insufficiency (Matsumoto *et al.*; 2003). Taken together, these experiments suggest that SOCS3 may play an important role in the regulation of fetal liver erythropoiesis (Roberts *et al.*; 2001; Wormald and Hilton; 2007). Given that Epo signalling is required for erythropoiesis, it is possible that SOCS3 modulates this process by attenuating Epo signalling (Sasaki *et al.*; 2000).

1.4 Cytokines

Cytokines are soluble glycoproteins, which mediate intercellular communication by binding to their specific receptors on the surface of target cells and transducing a signal to the nucleus to induce transcription of a specific set of genes. One way to classify cytokines is based on their structural and functional properties and the receptor molecules they bind to. Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), utilise receptors with intrinsic tyrosine kinase activity, termed receptor tyrosine kinases (RTKs) (Hubert; 2007). Conversely, members of the transforming growth factor-β (TGF-β) superfamily utilise receptors with serine/threonine kinase activity (Shimanuki *et al.*; 2007). Other subsets of cytokines include the TNF family. Sometimes the term "cytokine" is used only for factors whose main targets are the hematopoietic cells, and are thus called the hematopoietic cytokines. The hematopoietic cytokines, together with structurally related cytokines acting for example in the neural system, are collectively called class I cytokines. This class includes interleukins (IL) -2, -3, -4, -5, -6, -7, -9, -11,-12, -13,

15, -21, -23, erythropoietin (EPO), thrombopoietin (TPO), prolactin (PRL), growth hormone (GH), thymic stromal lymphopoietin (TSLP), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), neurotrophin-1 (NNT-1)/B-cell-stimulating factor-3 (BSF-3), and colony-stimulating factors for granulocytes (G-CSF) and granulocyte-macrophages (GM-CSF) (John *et al.*; 2006). Interferons (IFN) and IL-10 constitute an additional family termed the class II cytokine family. IL-19, -20, -22, and -24 have been recently identified as IL-10-related class$_{II}$ cytokines (Sabat *et al.*; 2007). The class I and class II cytokines share an α-helical three-dimensional structure. Class I cytokines typically consist of four α-helices, designated from A to D and connected by short loops (Huyton *et al.*; 2007). The class II cytokines, IFN-γ and IL-10, contain 6 α-helices instead of 4. The helical cytokines function usually as monomers, IFN-γ and IL-10 are dimers (Oliveira Neto *et al.*; 2008). The receptors utilised by type I and II cytokines are structurally related and form the hematopoietic receptor superfamily. Importantly, receptors of the hematopoietic receptor superfamily share common mechanisms for signal transduction (Nishimoto and Kishimoto; 2008).

1.4.1 IL-6

IL-6 plays an active role in immunity, bone metabolism, reproduction, arthritis, and in the development of neoplasia. Dysregulation of IL-6-type cytokine signalling contributes to the onset and maintenance of several diseases such as multiple myeloma, inflammatory bowel disease and rheumatoid arthritis (Nishimoto and Kishimoto; 2006). Moreover, IL-6 plays a significant role in the acute phase inflammatory response. IL-6 stimulates the acute phase reaction that enhances the innate immune system and protects against tissue damage. It results in the release of certain proteins, called "acute phase proteins", into the plasma by liver cells and induces a decrease in the rate of synthesis of other proteins. Specifically, IL-6 increases the synthesis of the two major acute phase proteins, C-reactive protein (CRP), which increase the rate of phagocytosis of bacteria, and serum amyloid (SAA) by regulating the rate of gene transcription (Dziedzic; 2008).

IL-6 also increases the synthesis of fibrinogen, an important clotting agent albumin and transferrin levels are also decreased in the presence of IL-6 (Ohta *et al.*; 2004; Marquardt *et al.*; 2005). Not surprisingly, knockout of the IL-6 gene has severe effects on the immune system, including a major decrease in the acute phase immune reaction and production of IgA antibodies (Benihoud *et al.*; 2000). On the other hand, overexpression of the IL-6 gene can lead to the substantial polyclonal proliferation of plasma cells. Lack of gene regulation can lead to autoimmune disease and many lymphoid malignancies, including multiple myeloma. An uncontrolled or defective production of this protein most often leads to disease and is involved in the pathogenesis of many disease and autoimmune disorder such as liver autoimmune disease. Furthermore, IL-6 is important for the development of specific immunological responses and also plays an important role in bone metabolism through the initiation of osteoclastogenesis and increasing osteoclast activity (Liu *et al.*; 2006).

1.4.1.1 IL-6 structure

The average molecular weight of human IL-6 ranges between 21 to 28 KDa, depending on the post-translational processing, such as glycosylation. The full length IL-6 peptide comprises 212 amino acids (aa) from which a 28 aa hydrophobic signal peptide is cleaved off from the N-terminus resulting in a mature protein of 184 aa. IL-6 contains four conserved cysteine residues within a highly conserved central region. Additionally, the C-terminus appears to be critical for IL-6 activity, since deletion of four aa from the C-terminus abolishes IL-6 activity. In contrast, deletion of 28 aa from the N-terminus does not affect IL-6 activity (Maggio *et al.*; 2006).

1.4.1.2 IL-6 Receptors

IL-6 receptors can be subdivided into non-signalling α-chain and signal transducing subunits such as gp130. IL-6 first binds specifically to its respective α-receptor subunits. The ectodomain of the receptors involved in IL-6 signalling comprise an array of FNII-like and Ig-like domains (Heinrich *et al.*; 2003) (Figure 1.3).

Mutagenesis studies have identified distinct areas on the surface of the cytokine, which specifically interact with the respective receptors. Common to all IL-6-family members is the site II that interacts with the cytokine binding module (CBM) of gp130 (Nishimoto and Kishimoto; 2008).

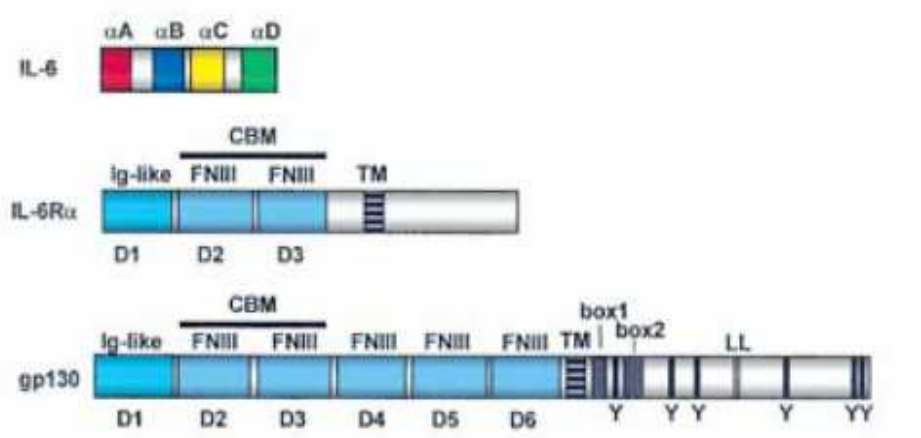


Figure 1.3 Receptor complexes of IL-6-type cytokines. For gp130 the box1 and box2 regions, as well as the dileucine motif (LL, Leu786-Leu787), are highlighted (Heinrich *et al.*; 2003).

Cytokine receptors are single trans-membrane glycoproteins that have a conserved extracellular domain and three short conserved motifs in the intracellular regions. The intracellular region of cytokine receptors do not have intrinsic catalytic activity, but are linked via ligand binding to activation of signal transduction by associating with a number of signalling proteins (Heinrich *et al.*; 2003). The cytokine receptors have box1 and box2 motifs (Haan *et al.*; 2006). Box1 is located within the first 20 amino acids of the cytoplasmic domain. Box2 motif is characterised by a stretch of hydrophobic amino acids, which are followed by several charged amino acids (Haan *et al.*; 2002; Heinrich *et al.*; 2003).

The membrane-distal region of cytokine receptors also mediate essential signalling functions of receptors and contain tyrosine residues that become phosphorylated upon cytokine stimulation. These serve as docking sites for Src homology2 (SH2) domain-containing signalling proteins. IL-6 receptors are membrane proteins that belong to the cytokine receptor class I family. This receptor family is defined by the presence of at least one cytokine binding module (CBM) consisting of two fibronectin-type-III-like domains of which the N-terminal domain contains a set of four conserved cysteine residues (Aasland *et al.*; 2003) and the C-terminal domain a WSXWS motif (Heinrich *et al.*; 2003) (Figure 1.3).

Moreover, IL-6R has an IgG-like domain located at the N-terminus and three additional membranes proximal fibronectin typeIII like domain. In addition to the membrane-bound receptor, a soluble form of the IL-6R (sIL-6R) has been purified from human serum and urine. This soluble receptor binds IL-6 with an affinity similar to that of the cognate receptor and prolongs its plasma half-life (Michalopoulou *et al.*; 2004). More importantly the sIL-6R/IL-6 complex is capable of activating cells via interaction with membrane-bound gp130. This is in contrast to the function of most soluble cytokine receptors that bind their ligand to antagonise cellular signalling by preventing the interaction of the cytokine with their respective plasma membrane-bound receptor (Jones *et al.*; 2005). Soluble IL-6 receptors present on hematopoietic progenitor cells, Kaposi's sarcoma cell lines and synovial fibroblasts can form a ligand receptor complex with IL-6 which stimulates a variety of cellular responses including proliferation, differentiation and activation of inflammatory processes (Peake *et al.*; 2006; John *et al.*; 2007). Moreover, gp130 is used not only by the IL-6 receptor but also by the receptors for other members of IL-6 family LIF and OSM (Nishimoto and Kishimoto; 2008).

SIL-6R performs an important role in the regulation of IL-6 responses and consequently disease progression. Although changes in sIL-6R concentration have

been determined in numerous clinical disorders, high IL-6 concentrations hve been documented in the serum and synovial fluid of rheumatoid arthritis and juvenile rheumhatoid arthritis patients (Pignatti *et al.*; 2003; Peake *et al.*; 2006). Through a series of *in vitro* approaches, sIL-6R has been implicated in a variety of cellular conditions typically associated with arthritis, such as the severe destruction of cartilage and bone (Pignatti *et al.*; 2003).

Examination of both IL-6 and sIL-6R concentrations in synovial fluid from arthritic patients showed that the extent of joint destruction correlated with the increased concentration of these mediators. Furthermore, synovial fluids from rheumatoid arthritic patients containing high levels of IL-6 and sIL-6R promoted osteoclast like cell formation when added to cocultures of osteoblastic cells and bone marrow cells (Peake *et al.*; 2006).

1.4.1.3 Signalling of IL-6-type cytokines

IL-6 utilises tyrosine kinases of the JAK family and transcription factors of the STAT family as mediators of signal transduction. gp130 is constitutively associated with Tyr kinases JAK1, JAK2 and TYK2. Several phosphotyrosine residues of gp130 are docking sites for STATs with matching SH2 domains, particularly, STAT1 which bind to phospho Tyr^{701} in gp130 and STAT3 binds to phospho Tyr^{705} (Klein *et al.*; 2005). STATs then become Tyr-phosphorylated, form dimers and translocate to the nucleus, where they regulate transcription of target genes (Lim and Cao; 2006) (Figure 1.4).

Moreover, the tyrosine phosphatase SHP2 also binds to phosphotyrosine 759 motif of activated gp130 and counteracts receptor and STAT activation (Lehmann *et al.*; 2003). Also, Suppressor of cytokine signalling (SOCS) 3 is a potent IL-6-induced feedback inhibitor terminating IL-6 signal transduction is bind to the same site in gp130 and inhibits JAK activity (Fischer *et al.*; 2004).

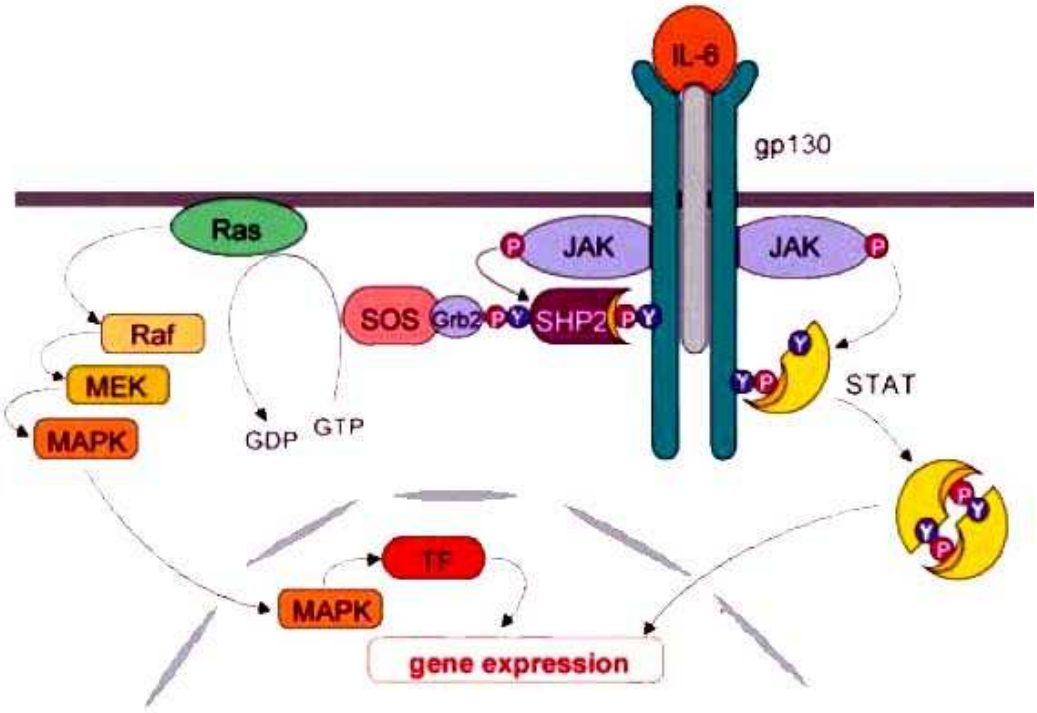


Figure 1.4 Schematic presentation of gp130 mediated activation of JAK/STAT pathway and MAPK / ERK cascade by IL-6 signalling (Heinrich *et al.*; 2003).

1.4.2 IFN alpha

The interferons (IFNs) represent proteins with anti-viral activity that is secreted from cells in response to avariety of stimuli. There are at least five classes of IFN alpha, beta, gamma, tau and omega. The interferons are divided into two groups designated type I and type II interferons. IFNγ is the only type II interferon, whereas the type I interferons consist of four major classes: IFN-*α*, IFN-*β*, IFN-*W*, IFN-*I*, and IFN-*K* (Caraglia *et al.*; 2005).

Type I IFNs have pleiotropic effects in both the innate and the adaptive immune responses. Recently, it has become clear that one of the key cells in the IFN-α physiological response is the natural IFN-producing cell (NIPC), also known as the

immature plasmacytoid dendritic cell PDC or precursors of type 2 DC (pDC2). PDCs differentiate into mature antigen-presenting DCs, which have a crucial role in T and B cell activation. NIPCs/PDCs were shown to be the major IFN-α producer in response to a wide range of agents, including bacteria and immune stimuli (Fitzgerald-Bocarsly and Feng; 2007). The pivotal role of IFN-α and NIPC/pDC in autoimmune disorders has also been demonstrated (Gottenberg and Chiocchia; 2007). Although IFN-γ production was thought to be restricted to T and NK cells, recent findings demonstrate that other cell populations of the DC lineage involved in antitumour defense produce IFN-γ, which mediates cancer cell apoptosis via tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induction (Taieb *et al.*; 2006) Type I and type II IFNs play a central role in host defenses not only against viruses but also against intracellular bacteria and parasites (Casanova and Abel; 2004). It has been clearly demonstrated that the lack of IFN receptors or defects in signalling pathways are responsible for inherited immunodeficiencies against mycobacteria (Jouanguy *et al.*; 1996; Newport *et al.*; 1996). Human primary immunodeficiencies of type I IFNs have been recently discovered (Casrouge *et al.*; 2006). They appear to be due to defects in either type I IFN production or in type I IFN responses.

Type I and II IFNs are secreted cytokines that regulate numerous biological activities associated with host defence and homeostasis. The JAK-STAT pathway is the primary signalling pathway for the transcriptional regulation of many IFN-stimulated genes (ISGs). Following high-affinity binding to their specific cognate receptor complexes, type I and type II IFNs transduce signals through activation of receptor-associated JAKs. Binding of the type I IFNs (IFN-*α*, IFN-*β*, IFN-*W*, IFN-*I*, and IFN-*K*) to their cognate receptor complex induces association of the two receptor chains, IFNAR-1 and IFNAR2-c (Domanski *et al.*; 1997; Brierley and Fish; 2002), which leads to the phosphorylation of tyrosine residues located in the intracellular domain of each receptor chain. These tyrosine phosphorylation events are thought to be carried

out by TYK2 and JAK1, which are themselves activated by tyrosine phosphorylation (Uze *et al.*; 2007) .

Similarly, the type II receptor complex comprises two receptor chains, IFNGR-1 and IFNGR-2. Binding of the type II IFN for example, IFN-γ to its receptor complex leads to the phosphorylation and activation of JAK1 and JAK2. Upon activation, the JAKs phosphorylate specific tyrosine residues within the intracellular domains of the receptor subunits (Caraglia *et al.*; 2005). These phosphorylated residues serve as docking sites for STATs (Uze *et al.*; 2007). Once recruited to the receptor complex, the activated JAKs phosphorylate a single tyrosine residue within the C-terminus of the STAT proteins as described previously. The phosphorylated and activated STATs form both homodimeric and heterodimeric complexes that translocate to the nucleus and bind specific DNA sequences within the promoter regions of ISGs to initiate transcription (Wesoly *et al.*; 2007).

1.4.2.1 IFN-inducible STAT complexes

Type I IFN binding to its receptor induces the dimerisation of IFNAR-1 and IFNAR-2c receptor chains and the subsequent activation of the receptor-associated JAKs (Figure 1.5). The IFNAR-1 chain associates with Tyk2, and the IFNAR-2c chain associates with JAK1. Once activated, Tyk2 phosphorylates tyr^{466} on IFNAR-1, generating a docking site for STAT2 by means of its SH2 domain (Yan *et al.*; 1996; Campbell; 2005). Tyk2 then phosphorylates STAT2 on Tyr^{690}, which serves as a docking site for STAT1 (Wesoly *et al.*; 2007). Subsequently, STAT1 itself is phosphorylated on Tyr^{701}. This activated STAT1 and STAT2 heterodimer dissociates from the receptor complex and localises to the nucleus. Only the intracellular domain of the IFNAR-2c chain is necessary for mediating the docking and phosphorylation of STAT protein as well as the formation of STAT complexes (Colamonici *et al.*; 1995; Kotenko *et al.*; 1999).

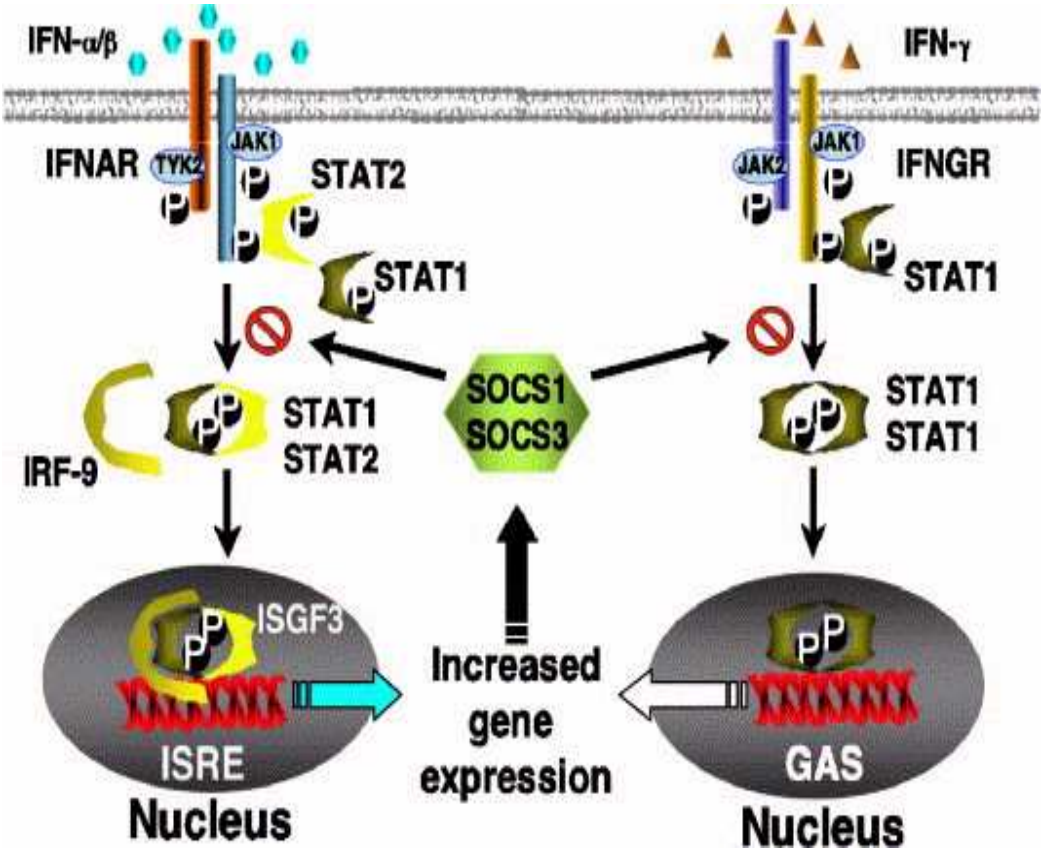


Figure 1. 5 Primary JAK/STAT pathways involved in cellular signalling by type I and type II IFNs.

Binding of either class of IFN to its receptor leads to activation of receptor-associated JAK proteins that phosphorylate tyrosine residues on the intracellular domains of the receptor. This is followed by the docking of STAT molecules to the receptor that are in turn phosphorylated at tyrosine residues by the JAKs. The activated STATs dissociate from the receptor chain and form dimers that translocate to the nucleus and bind to specific DNA recognition elements to modulate gene transcriptional activity. For the type I IFNs, both STAT1 and STAT2 are activated to form a heterodimer that associates in the nucleus with a third molecule, IRF-9. This trimolecular complex (also called ISGF3) binds to the interferon-stimulated response element (ISRE) (Campbell; 2005).

Within the IFNAR-2c intracellular domain, only a single tyrosine residue at either position 337 or 512 is required for a full IFN response (Velichko *et al.*; 2002). STAT2 associates with the IFNAR-2c subunit, whereas STAT1 binds only the IFNAR-2c-STAT2 complex, suggesting that STAT2 provides a recruitment site for the SH2 domain of STAT1, linking it to the receptor complex (Nadeau *et al.*; 1999; Campbell; 2005). This IFNAR-2c-STAT2 association does not depend on the tyrosine phosphorylation of the receptor or the STAT protein. Type I IFN induces the activation of all members of the STAT family, namely, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Wesoly *et al.*; 2007). Although numerous different types of STAT-containing complexes are formed, STAT2 is an essential component of type I IFN signalling (Paulson *et al.*; 1999). Notably, IFNs-α/β induce the formation of the ISGF3 complex, comprising STAT1, STAT2, and a DNA-binding adapter protein of the IFN regulatory factor (IRF) family, IRF-9 (p48 or ISGF3γ) (Genin *et al.*; 2003).

Upon nuclear import, STAT1 and IRF-9 of this ISGF3 complex bind the IFN-stimulated response element (ISRE), AGTTTN$_3$TTTC, to initiate gene transcription. Within this complex, STAT2 does not contribute to DNA binding but provides a potent transactivation domain (TAD) (Paulson *et al.*; 1999). Type I IFNs also induce the formation of other STAT-containing complexes: STAT1, STAT3, and STAT5 homodimers as well as STAT1 and STAT3 hetrodimers (Wesoly *et al.*; 2007). These homodimers and heterodimers bind palindromic so-called ‘‘GAS’’ sequences, TTCN$_3$GAA, designated GAS, located in the promoters of a different subset of ISGs. Type II IFN signalling primarily activates the STAT1 transcription factor (Figure1.5). The two chains of the type II IFN receptor, IFNGR-1 and IFNGR-2, associate prior to ligand binding. The biologically active form of IFN-γ is a noncovalent homodimer that binds its receptor complex in a 2:2 ratio (Walter *et al.*; 1995; Campbell; 2005).

In the case of IFN-γ binding, the intracellular domains of the receptor complex undergo a conformational change upon IFN-γ binds that induces the

autophosphorylation and activation of JAK2 and subsequent JAK1 transphosphorylation by JAK2 (Chang *et al.*; 2004). Activation of JAK1 phosphorylates tyrosine residue 440 within the intracellular domain of the two IFNGR-1 chains. STAT1 proteins associate with the IFNGR-1 chains by means of SH2-phosphotyrosyl interactions and are subsequently phosphorylated on Tyr^{701} (Hashemite *et al.*; 2004). The activated STAT1 proteins dissociate from the receptor complex and form STAT1 homodimers. Once in the nucleus, these STAT1 dimers bind GAS elements and induce gene transcription. These IFN-inducible STAT proteins thus permit direct transmission of signals from the cell surface to the nucleus, resulting in the generation of IFN-mediated biological responses (Campbell; 2005; Wesoly *et al.*; 2007).

1.4.3 Leptin Biology

Leptin is a multifunctional cytokine that plays a key role in the regulation of food intake and energy expenditure. The discovery of leptin at the end of 1994 (Zhang *et al.*; 1994) opened up a whole new perspective to study the role of adipocyte-derived factors in energy balance and homoeostasis (Fruhbeck *et al.*; 2001). The 16 kDa non-glycosylated polypeptide product of the *ob* gene is mainly produced and secreted by fat cells in proportion to fat mass to signal the repletion of body energy stores to the hypothalamus (Banks; 2004). Leptin exhibits striking structural similarities to members of the class I cytokine family, including LIF, CNTF, OSM and CT-1, as well as IL-6, IL-11 and IL-12 (Fruhbeck *et al.*; 1998; John *et al.*; 2006). Both the crystal structure and NMR studies of leptin have revealed that the protein adopts a cytokine fold similar to that exhibited by the short-helix subfamily of cytokine folds (Zhang *et al.*; 1997). The three-dimensional structure of the 167-amino-acid leptin molecule is based on four antiparallel α-helices, connected by two long crossover links and one short loop arranged in a left-handed helical bundle, which forms a two-layer packing. A disulphide bond between two cysteine residues (Cys96 and Cys146) of the C-terminus of leptin and the beginning of one of the loops has been shown to

be important for folding and receptor binding, as mutation of either of the cysteine residues renders the protein biologically inactive (Prolo *et al.*; 1998).

Circulating leptin concentrations have been reported to correlate closely with indvidual BMI (body mass index) and the total amount of body fat (Fruhbeck *et al.*; 1998; Strocchio *et al.*; 2007). Although leptin is mainly produced and secreted into the bloodstream by white adipocytes, this is not the only source of the hormone. Placenta (Valuniene *et al.*; 2007), gastric mucosa (Francois *et al.*; 2008), bone marrow (Isaia *et al.*; 2005), mammary epithelium (Motta *et al.*; 2007), skeletal muscle, pituitary (Tipsmark *et al.*; 2008), hypothalamus and bone have also been shown to be able to produce small amounts of leptin in certain circumstances (Masuzaki *et al.*; 1997; Bado *et al.*; 1998; Morash *et al.*; 1999). Based on an almost ubiquitous distribution of receptors, leptin has been reported to play a role in a diverse range of physiological functions both in the central nervous system and at the periphery (Fruhbeck; 2001; Fruhbeck; 2002; Bjorbaek and Kahn; 2004). Its functions encompass metabolism, reproduction, immunity, cardiovascular pathophysiology, respiratory function, wound healing, as well as in growth and development (Gainsford *et al.*; 1996; Holness *et al.*; 1999).

1.4.3.1 Leptin receptor

The hormone leptin is encoded by *Ob* gene. Leptin acts through its receptor (*db* gene), which has six isoforms (from ObRa to ObRf) (Malendowicz *et al.*; 2006). Only one of them the leptin receptor b (ObRb), has full signalling capabilities and is able to activate the JAK/STAT pathway. Like all other class I cytokines receptors, ObRb lacks any intrinsic kinase activity, and uses cytoplasmic-associated JAKs to signal downstream. Leptin binding results in formation of a dimmer receptor complex leading to trans-phosphorylation and activation of the JAKs (Kloek *et al.*; 2002). These then phosphorylate tyrosine residues (Tyr^{974}, Tyr^{985}, Tyr^{1138} and Tyr^{1077}) in the cytosolic domain of the receptor, which provide binding sites for signalling molecules

including SHP-2 and members of the STAT family, which are also subject to JAK-mediated phosphorylation activation (Baumann *et al.*; 1996; Bjorbaek and Kahn; 2004).

The full-length ObRb receptor contains several cytoplasmic sequence elements that are required for subsequent signalling events. ObRb binds JAK2 constitutively (Ghilardi and Skoda; 1997; Bjorbaek and Kahn; 2004) and, like other cytokine receptors, it contains a highly conserved, proline-rich box1 between intracellular a.a.'s 6-17 and two putative less conserved box2 motifs between intracellular a.a.'s 49-60 and 202-213 (Ghilardi and Skoda; 1997). While Box1 and box2 motifs are thought to recruit and bind JAKs (Haan *et al.*; 2006), it has been demonstrated that only box1 and the immediate surrounding amino acids are essential for JAK activation. Although an intact box2 motif is not required to activate JAK activity (Bahrenberg *et al.*; 2002), the pivotal STAT signalling pathway cannot be induced without box2. Forming homodimers and showing signalling capabilities with mutated box2 motfs, ObR can be classiflied as a member of the growth hormone receptor (GHR) subfamily (Fruhbeck; 2006). For downstream signalling events, tyrosine residues at positions 985 and 1138 are needed to provide docking sites for subsequent signalling molecules with SH2 domains such as STATs and SHP2 (Banks *et al.*; 2000; Heshka and Jones; 2001).

1.5 Adenosine receptors

Adenosine receptors belong to the G protein-coupled 7 transmembrane superfamily of cell surface receptors and include A_1, A_{2A}, A_{2B}, and A_3 subtypes. The interest in The realisation that the psychostimulatory effects of caffeine are largely due to antagonism of brain-tissue adenosine receptor signalling (Fredholm; 1995) further stimulated interest in adenosine receptors system. A better understanding of the purinergic receptors and their intracellular signalling pathways lead to the classification of adenosine receptors according to the rank order of potencies of agonists with respect to the intracellular production of cAMP. ARs transduce their

signal by heterotrimeric G-proteins that can either stimulate (Gs) or inhibit (Gi) adenylyl cyclase, the enzyme that catalyses the formation of cAMP (Sitkovsky *et al.*; 2004). The cloning of four AR subtypes (A_1, A_{2A}, A_{2B}, A_3) (Olah and Stiles; 1995; Palmer and Stiles; 1997) helped to prove that high-affinity A_{2A} and low-affinity A_{2B}AR activate adenylyl cyclase, whereas high-affinity A_1 and high-affinity A_3ARs inhibit it. Accordingly, when immune cells acquire the expression of multiple adenosine receptors, they will be recruited in a stepwise manner with Gi-coupled A_1AR activation initially at very low adenosine levels, followed by the stimulation of G_s-coupled A_{2A} and A_{2B}ARs, and finally by G_i-coupled A_3AR (Fredholm *et al.*; 2001; Sitkovsky *et al.*; 2004).

1.5.1 A_{2A}AR

The A_{2A}AR is highly expressed within most cells of the immune system, platelets, heart, lung and endothelium (Fredholm *et al.*; 2001). Classically, the A_{2A}AR signals via the G_s family of G proteins leading to an activation of adenylyl cyclase and the generation of cAMP. However, it has been shown that there may be cell type-specific patterns of A_{2A}AR-activated signalling. A_{2A}ARs have been cloned from several species, including dog (Libert *et al.*; 1990), rat (Fink *et al.*; 1992) and man (Furlong *et al.*; 1992) and show 84% sequence identity between rat and human forms.

Adenosine-induced vasodilatation is thought to involve both A_{2A}AR and A_{2B}ARs (Ralevic and Burnstock; 1998; Sitkovsky *et al.*; 2004; Fredholm; 2007). The A_{2A}AR is expressed in ECs and its activation may lead to activation of nitric oxide synthase (Lin *et al.*; 2007). A direct effect of adenosine on arterial smooth muscle cells has also been proposed to contribute to vasodilatation (Kleppisch and Nelson; 1995; Tamajusuku *et al.*; 2006). In addition to increasing local blood flow, the extracellular accumulation of adenosine triggers signalling cascades that enable oxygen-deprived tissues to cope with both the short-term (Fredholm; 1997; Picano and Abbracchio; 1998) and the long-term effects of oxygen deprivation (Sexl *et al.*; 1995; Sexl *et al.*; 1997). ECs proliferation is a consequence of the activation of ERK via A_{2A}AR. This

is also evoked by activation of β2-adrenergic receptors. It is surprising that stimulatory receptors linked to cAMP formation activate ERK since cAMP inhibits proliferation in many cells. Indeed, signalling to ERK in endothelial cells is independent of cAMP, but through an unidentified pathway that involves the small G-protein Ras (Barthomeuf *et al.*; 2004). In immortalised cell lines, A_{2A}AR activation leads to ERK phosphorylation (Seidel *et al.*; 1999; Schulte and Fredholm; 2000). However, the signal cascade that links the A_{2A}AR to ERK differs between cell types. In CHO cells, cAMP formed by stimulating A_{2A}AR activates Rap1 through a cAMP-dependent exchange factor (EPAC). Rap1 then associates with B-raf, but this is insufficient for ERK activation in CHO cells. Alternative intermediary candidates are protein kinase A (PKA) and members of the Src nonreceptor tyrosine kinase family because A_{2A}AR-dependent ERK activation can be blocked by appropriate inhibitors (Seidel *et al.*; 1999).

The coupling of the A_{2A}AR to its cognate G protein G_s displays two unusual features. Firstly, adenylyl cyclase activation rates are incompatible with the collision coupling model that adequately describes the kinetics of normal receptor/G_s interaction; nevertheless, in reconstitution experiments, the receptor can actually be shown to use G_s as a substrate (Nanoff *et al.*; 1994; Fredholm; 2007). Secondly, in membranes, addition of guanine nucleotides fails to induce dissociation of the ternary HR complex of agonist (H), receptor (R) and G protein (G), such that the agonist remains trapped in the high-affinity state. Brief incubation of the membranes with trypsin results in proteolytic cleavage of the receptor and restores guanine nucleotide regulation of agonist binding (Nanoff *et al.*; 1991). Proteolysis reduces the apparent molecular mass of the receptor by some 8 kDa; this is most likely accounted for by cleavage of the extended C-terminus because of the small size of the N-terminus. Taken together, these data suggest a role for the C-terminus in regulating the coupling properties of the A_{2A}AR. Since the majority of the C-terminal tail itself is dispensable for a productive interaction with the G protein, this domain may represent the anchor for a modulatory component (Grieco *et al.*; 2003) .

The A_{2A}AR displays a considerable degree of constitutive activity and this is independent of the cell line and the receptor expression level (Ledent *et al.*; 1992; Nanoff and Stiles; 1993; Yang *et al.*; 2005). Although both properties seem to reflect a preference of the receptor to remain in the active, G protein-coupled conformation, constitutive activity and guanine nucleotide refractoriness can be dissociated. The extended C-terminal domain of the receptor is primarily responsible for constitutive activity. This is shown in truncated receptors which lose constitutive activity and in chimeric receptor constructs where cytoplasmic loop 3 and the C-tail of the A_{2A}ARs have then introduced into the A_1AR and which become spontaneously active (Tucker *et al.*; 2000). However, the inability of guanine nucleotides to convert the receptor to the low-affinity conformation is preserved in the C-terminally truncated receptor. It seems that the guanine nucleotide refractoriness of the membrane-bound receptor is solely due to coupling to G_s, the G_s selectivity being specified by cytoplasmic loop 3 (Olah; 1997; Tucker *et al.*; 2000).

Given the apparent link of constitutive activity with an intact C-terminus, this domain deserves closer examination. The C-terminus is rich in serine/threonine residues (12 of 122 residues) and, in cell experiments, is rapidly phosphorylated following activation of PKC. However, mutations that remove the consensus sites for PKC-dependent phosphorylation do not affect the ability of the receptor to serve as a substrate for phosphorylation. It is therefore likely that PKC isoforms do not directly phosphorylate the receptor but that they activate an intermediary kinases (Palmer and Stiles; 1999). In addition, it is worth noting that the C-terminus of the A_{2A}AR is enriched with proline residues: 22 prolines are present in the human A_{2A}AR, 11 of which are interspersed in the first 311 amino acids. The remaining 11 prolines are found in the last 100 amino acids with a cluster of four occurring in an acidic stretch at positions −10 to −18 removed from the C-terminus and a second cluster of five prolines between positions 341 and 357. A sequence comparison of the A_{2A}AR shows a high degree of conservation in man, dog and guinea pig. However, the C-terminus

of the rat receptor diverges substantially. Regardless of this discrepancy between phylogenetic relation and species orthologues of the receptor, the proline-rich stretches are obviously indicative of a potential interaction with SH3 domains. In other instances, SH3 domain-containing proteins have indeed been found to bind to G protein-coupled receptors (Oldenhof *et al.*; 1998). A recent study has provided a functional link between the A_{2A}AR and the actin cytoskeleton. Direct interaction between α-actinin and A_{2A}AR was observed suggesting that agoinst-mediated clustering and internalisation of the A_{2A}AR regulated by its C-terminus is dependent on an intact α-actinin/actin network (Burgueno *et al.*; 2003).

1.5.1.1 The A_{2A}AR as anti-inflammatory GPCR

Multiple in *vivo* and in *vitro* studies suggest a potent anti-inflammatory role of the A_{2A}AR. Early reports indicated that A_{2A}AR activation could inhibit superoxide release from guinea pig and human eosinophils stimulated with opsonised zymosan (Yukawa *et al.*; 1989). Furthermore, the A_{2A}AR agonist NECA inhibits Fc-γ R-mediated phagocytosis and superoxide generation in polymorphonuclear leukocytes (PMN) which inhibited by the AR antagonist 8-ρ-sulphenyltheophylline (Salmon and Cronstein; 1990). It has been reported that adenosine or NECA concentrations sufficient to inhibit the generation of ROS decreases adhesion of fMLP-induced PMNs to endothelial cells (Cronstein *et al.*; 1992). This reduction was found to be due to the inhibition of $\alpha_M\beta_2$ integrin expression (Wollner *et al.*; 1993).

The potent anti-inflammatory effect of the folate drug methotrexate (MTX) is also adenosine-dependent (Chan and Cronstein; 2002). Low concentrations of MTX reduce leukocyte accumulation in carrageenan-inflamed air pouches (Cronstein; 1994). This effect was completely reversed by an A_{2A}AR antagonist DMPX, suggesting that adenosine acting at an A_{2A}AR subtype mediated the protective effect. The same study also demonstrated that inhibition of adenosine kinase reduced leukocyte accumulation, an effect that was completely reversed by co-injection of adenosine deaminase (Cronstein *et al.*; 1994).

A_{2A}AR gene-deficient mice have proved to be an important tool in studying the anti-inflammatory effects of the A_{2A}AR and in establishing the critical role of these receptors in several models of immune-mediated tissue damage (Ledent *et al.*; 1997; Ohta and Sitkovsky; 2001). The absence of A_{2A}AR on immune cells causes the cessation of cAMP production in activated immune cells, allowing uninterrupted tissue damage (Sitkovsky *et al.*; 2004). It has been reported that the A_{2A}AR has a role in wound healing as mice lacking the A_{2A}AR form less dense granular tissue and fewer blood vessels during wound repair and accumulate fewer leukocytes in response to inflammatory stimuli (Montesinos *et al.*; 2002).

The anti-inflammatory role of A_{2A}AR has driven the development of selective agonists to exploit its therapeutic potential (Lappas *et al.*; 2005). Most A_{2A}AR-selective agonists are 2-substitution of the non-selective AR agonist NECA, such as CGS21680, HENECA (Cristalli *et al.*; 1992; Rebola *et al.*; 2003), ATL-146e (Chang *et al.*; 2007) and MRE-0470 (Glover *et al.*; 2001). Selective activation of the A_{2A}AR has been shown to have significant protiective effects in several models of inflammation, including a decrease in neutrophil transmigration into the cerebrospinal fluid in patients with endotoxin-stimulated meningitis, attenuation of the inflammation-induced increase in the blood-brain barrier permeability (Sullivan *et al.*; 1999), inhibition of oxygen radical production (Thiel *et al.*; 2003) and inhibition of the production of cytokines such as TNFα (Majumdar and Aggarwal; 2003). In addition, ATL-146e reduced joint destruction caused by septic arthrosis and CGS-21680 can regulate HIV-1 transactivation regulating protein (Tat)-induced inflammatory responses (Cohen *et al.*; 2004; Fotheringham *et al.*; 2004).

In mouse models of ischaemia/resperfusion injury, co-reperfusion of the A_{2A}AR-selective agonist ATL-146e decreased liver injury by 90%. Co-administration of the A_{2A}AR-selective antagonist ZM241385 attenuated this effect indicating the receptor specificity of the response (Day *et al.*; 2004). In addition, the A_{2A}AR was shown to

protect from concanavilin A-induced liver injury *in vivo* (Ohta and Sitkovsky; 2001). *In vitro* studies suggests the anti-inflammatory response is due to a reduction in the expression of cytokine and chemokines from cells expressing the A_{2A}AR (Bshesh *et al.*; 2002). THP-1 cells treated with LPS increased the expression of A_{2A}AR and adenosine. Further treatment of the cells with an A_{2A}AR-selective agonist CGS21680, decreased LPS-stimulated TNFα production in a time and dose-dependent manner. This inhibitory pathway involves A_{2A}AR-mediated activation of PKA and phosphorylation of cAMP response element binding protein (Bshesh *et al.*; 2002) .

One of the most well-defined protective effects of AR activation is inhibition of pro-inflammatory responses. While numerous studies have shown that activation of A_{2A}ARs on neutrophils represents a critical mechanism by which adhesion is blocked, selective activation of A_{2A}AR in ECs from multiple origins also inhibits leukocyte-EC interaction. For example, Bouma *et al.* (1996) demonstrated that pre-treatment with either adenosine or the non-selective AR agonist 2-chloroadenosine inhibited the release of IL-6 and IL-8 from IL-1β and LPS-activated HUVECs. AR activation also reduced the potency with which TNF-α could induce the cell surface expression of E-selectin and VCAM-1 with no effect on ICAM-1 induction (Bouma *et al.*; 1996). Analysis of these anti-inflammatory effects suggests the involvement of one or both of the A_{2A}AR subtypes. Recent work employing more selective drugs has revealed that A_{2A}AR activation is sufficient to inhibit E-selectin induction both *in vivo* (McPherson *et al.*; 2001) and *in vitro* (Sands *et al.*; 2004). Interestingly, while A_{2A}AR stimulation is capable of inhibiting induction of VCAM-1 *in vitro*, this effect cannot be mimicked simply by elevation of intracellular cAMP levels (Bouma *et al.*; 1996), suggesting that the receptor must activate at least one additional signalling pathway in ECs to mediate this effect. A_{2A}ARs on ECs are potentially useful targets for drugs aimed at alleviating endothelial dysfunction. Administration of agonists should enhance the protective effects of receptor activation and reverse the excessive inflammation associated with disease progression. Several observations support the potential utility of the A_{2A}AR in this regard. First, administration of the selective

agonist ATL-146e reverses the accumulation of VCAM-1, P-selectin and ICAM-1 observed in a carotid ligation model of vascular injury (McPherson *et al.*; 2001), although the ability of A_{2A}AR activation to inhibit leukocyte and platelet activation may also indirectly inhibit EC activation by attenuating leukocyte-EC and platelet-EC interaction. Second, it has been demonstrated that potentiation of A_{2A}AR-activated signalling pathways in vascular ECs by adenovirus-mediated human A_{2A}AR gene transfer is sufficient to block E-selectin induction and monocyte adhesion to TNFα-stimulated HUVECs even in the absence of an A_{2A}AR-selective agonist (Sands *et al.*; 2004). This is an important observation as it suggests that increasing flux through A_{2A}AR-activated signalling pathways in ECs can dramatically suppress the pro-inflammatory events associated with diseases, such as atherosclerosis and sepsis (Montesinos *et al.*; 2002) and rheumatoid arthritis (Montesinos *et al.*; 2000).

Blocking the effects of endogenous extracellular adenosine in wild type mice using selective A_{2A}R antagonists addressed possible caveats for interpreting experiments using genetically engineered mice. These experiments confirmed the conclusions derived from the studies of A_{2A}AR gene-deficient mice. Injection of A_{2A}AR antagonists exacerbates tissue damage by sub-threshold doses of inflammatory stimuli. In addition, control experiments confirmed that although extracellular adenosine-triggered cAMP accumulation was severely inhibited in A_{2A}AR-deficient mice, agonists at other G_s-protein-coupled receptors were still capable of inducing similar degrees of elevation of cAMP in wild-type and A_{2A}AR-deficient mice (Lukashev *et al.*; 2003). Other studies confirmed that A_{2A} adenosine receptor-deficient mice did not have unanticipated spontaneous mutations in the cAMP-signalling, inflammation-inhibiting pathway downstream of the A_{2A}AR (Ohta and Sitkovsky; 2001).

1.6 Ubiquitination

Ubiquitination/ubiquitylation refers to the post-translational modification of a protein by the covalent attachment (via an ε-amino isopeptide bond) of one or more Ub mnomers. Ub is a highly conserved small (8 kDa) regulatory protein that is ubiquitous in eukaryoyes (Hershko and Ciechanover; 1998; Hochrainer and Lipp; 2007). The most well characterised function of Ub is labeling proteins for proteasomal degradation (Hochrainer and Lipp; 2007). Ubiquitination also controls the stability, function and intracellular localisation of a wide variety of proteins. Ubs covalently attach via an isopeptide bond to the ε-amino group of Lys residues on target proteins. This occurs through a three-step process involving Ub-activation (E1), Ub-conjugation (E2) and Ub-ligation (E3) enzymes (Hershko and Ciechanover; 1998; Gao and Karin; 2005). The types of Ub modifications that can form are diverse. In the simplest form, a single Ub molecule is attached, which is defined as monoubiquitylation (Hicke and Dunn; 2003). Alternatively, several Lys residues can be tagged with single Ub molecules, giving rise to multiple monoubiquitylation, also referred to as multiubiquitylation (Haglund *et al.*; 2003). Since Ub contains seven Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63), Ub molecules can form different types of chains in an iterative process known as polyubiquitylation (Pickart and Fushman; 2004). All seven Lys residues are possibly involved in chain formation in *vivo*, and Ub chains linked *via* Lys48 or 63 are the best characterised so far (Hicke *et al.*; 2005). It is clear that Lys48-linked poly-Ub chains represent a signal for proteasomal degradation of modified substrates. This discovery merited the award of a Nobel Prize in Chemistry 2004 and has been extensively reviewed (Hershko and Ciechanover; 1998). However, other types of Ub conjugates are involved in the regulation of different cellular processes independently of proteolytic degradation (Haglund *et al.*; 2003; Hicke and Dunn; 2003; Krappmann and Scheidereit; 2005).

1.6.1 Ub as an inducible and reversible signal

It is well established that protein ubiquitylation is induced by a vast variety of stimuli and upstream signalling events in cells. For example, various cell surface receptors become ubiquitylated upon stimulation with extracellular ligands (Hicke and Dunn; 2003). In addition, many cytoplasmic and nuclear proteins become ubiquitylated following their phosphorylation (Fiore *et al.*; 2003; Muratani and Tansey; 2003). Moreover, the functions of Ub ligases are tightly regulated by signal-induced mechanisms such as compartmentalisation, degradation, oligomerisation and post-translational modifications (Hershko and Ciechanover; 1998; Thien and Langdon; 2001; Dikic and Giordano; 2003). Regulation at this level is particularly important, since Ub ligases play a central role in substrate recognition and specificity. A second key feature of the ubiquitylation system is that Ub can be rapidly removed by deubiquitylating enzymes (DUBs), which serve to switch off the Ub signal or to shift between different modifications of the same Lys residue (Hershko and Ciechanover; 1998; Amerik *et al.*; 2006). Notably, ubiquitylation shares the two above-mentioned similarities with protein phosphorylation. In addition, both modifications are recognised by specific protein domains, providing a mechanism for translation of the Ub or phospho-specific signal to downstream effectors (Pawson *et al.*; 2001; Hicke *et al.*; 2005). Intriguingly, although it might not be universally required, phosphorylation is a signal that often precedes ubiquitylation of proteins either at the level of the E3 Ub ligase (e.g. Cbl) or the substrate protein (e.g. Eps15, Hrs and IκB). This shows that the two modifications are in tight cooperation in cells. A major difference between the two systems is that Ub is a chemically more complex molecule than phosphate, since it has a larger surface to interact with other proteins. The fact that Ub can form chains increases the complexity even further. In fact, the Lys63 and Lys48 chains have different conformations, with Lys63 chains being much more extended than those linked via Lys48, indicating that they likely have distinct targets and functions in the cell (Pickart and Fushman; 2004). All these features allow the ubiquitylation system to integrate and synchronise protein networks all the way from the cellular membrane to the nucleus.

1.6.2 Proteasomal degradation

Ub-mediated protein degradation provides a major mechanism for controlled proteolysis of targeted proteins. Ub conjugation to a substrate involves a cascade of at least three different enzymatic reactions (Figure1. 6). First, Ub is activated by E1, the Ub-activating enzyme, to form a high-energy thioester linkage between its C-terminal glycine residue and an active cysteine on the E1. Next, the thiol-linked Ub is transiently transferred to the next enzyme in the cascade E2, the Ub conjugating enzyme. Finally, an E3 ubiquitin ligase either transfers the activated Ub molecule from the E2 to a lysine residue on the substrate or facilitates the transfer of Ub from the E2 directly to the substrate (Hershko and Ciechanover; 1998; Gao and Karin; 2005).

The substrates marked with a Lys48-linked polyubiquitin chain are selectively targeted for 26S proteasome-mediated degradation (Wilkinson *et al.*; 2001). The consequences of Ub attachment depend upon how many Ub moieties are attached and the chain-linkage involved. Recognition for degradation by the proteasome involves polyubiquitin chains of at least four molecules in length (Thrower *et al.*; 2000) in which each Ub is linked via an isopeptide bound from the carboxy-terminus of one Ub to K48 on the adjacent Ub (Petroski and Deshaies; 2005). A proteomics screen of Ub conjugates in *Saccharomyces cerevisiae* found Ub modified at all seven lysine residues (Peng *et al.*; 2003). The 26S proteasome consists of the 20S core complex, composed of four stacked rings of seven subunits that contain the proteolytic sites in the central cavity (Groll *et al.*; 1997). and a multisubunit 19S regulatory particle that caps both ends of the 20S particle. The 19S particle, comprising a lid, a linker and a base, mediates the recognition of polyubiquitinated targeted proteins and promotes their unfolding in an ATP-dependent reaction. The base contains eight subunits (six ATPases of the AAA family and two large subunits, S2/Rpn1 and S1/Rpn2) and is connected to the lid by the S5a/Rpn10 protein (Ferrell *et al.*; 2000).

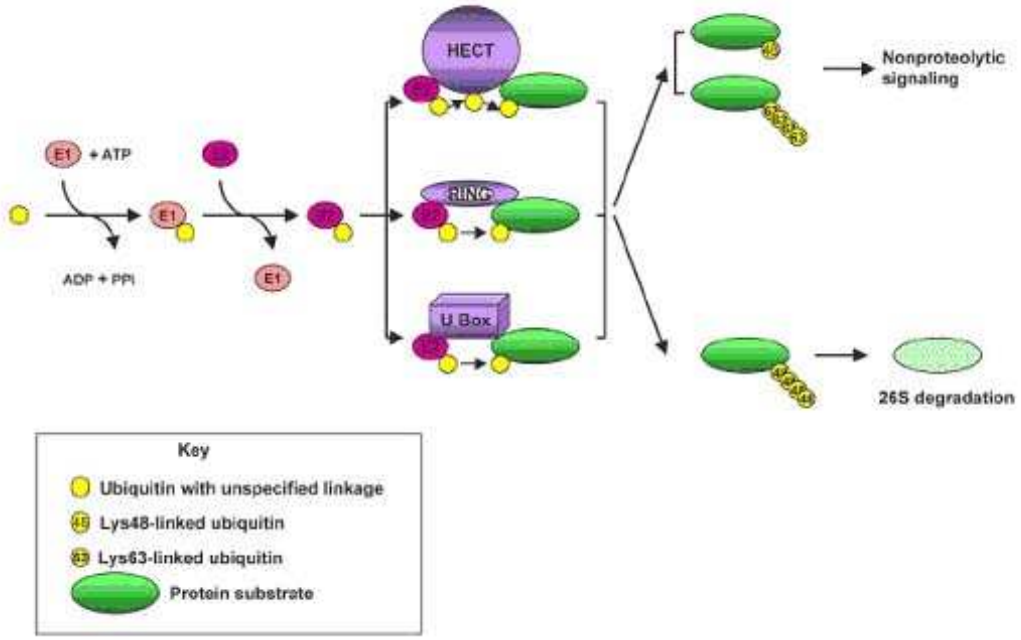


Figure 1. 6 Overview of the Ub Conjugation Pathway

Ub is first activated by a Ub-activating enzyme E1; activated Ub is then transferred to a Ub-conjugating enzyme E2; a Ub ligase E3 facilitates the transfer of Ub from E2 to the protein substrate. There are two major classes of E3 Ub ligases: proteins with a HECT catalytic domain and proteins with a RING finger adaptor domain. Additional E3s, such as those containing a U box, were recently described. Substrates marked with a Lys48-linked polyubiquitin chain are selectively targeted to 26S proteasome-mediated degradation, whereas certain substrates conjugated with mono- or multiubiquitins or Lys63-linked polyubiquitin chains are targeted for endocytosis or are enabled to engage in new protein-protein interactions. The polyubiquitin chain can be removed from the substrate by a DUBs (Gao and Karin; 2005).

1.7 E3 ligase and STAT degradation

Ubiquitination is implicated in the regulation of STATs. The ubiquitination of STAT1 has been reported (Kim and Maniatis; 1996), but the underlying molecular mechanisms remained unknown. Proteasome inhibitors were also shown to stabilise the tyrosine-phosphorylated forms of STAT4, STAT5, and STAT6 (Wang *et al.*; 2000). Recent findings have clarified the role of ubiquitination in the regulation of STATs and provided insights into the specificity of this modification.

Paramyxoviruses efficiently use the Ub pathway as a mechanism to escape the antiviral activities of IFNs (Horvath; 2004b). Paramyxoviruses are RNA viruses and include the human pathogens mumps, measles, and Nipah viruses (Nishio *et al.*; 2002). Their host evasion mechanisms are largely attributed to the V proteins. At least three V proteins function as E3 ligases with high specificity for STAT1, STAT2, or STAT3. The V proteins form a multisubunit E3 enzyme complex that shows homology to the SOCS-E3 complex. The current model of the E3 ligase suggests a complex that contains the V protein and the cellular V-interaction proteins (VIPs), as well as additional proteins, including DDB1 (an ultraviolet- damaged DNA binding protein) and members of the Cullin family, especially Cullin 4A (Cul4A). The E3 complex is termed VDC (for V/DDB1/CUL4A or V-dependent degradation complex) (Figure 1.7). VDC mediates STAT degradation using a combination of virus-encoded and cell-derived factors, where VIPs are the core degradation complex and the other cellular cofactors are responsible for the variations in V-protein target specificities (Ulane and Horvath; 2002).

Recently, SLIM, an Ub E3 ligase for Tyr-phosphorylated STATs, was identified, promoting the ubiquitination and degradation of STAT1 and STAT4. This is supported by the enhanced protein levels of STAT1 and STAT4 observed in SLIM-deficient mice (Tanaka *et al.*; 2005). SLIM also inhibited the Tyr phosphorylation of STATs, which is independent of its role in proteasomal degradation. There are two plausible interpretations. First, SLIM could act as an adaptor molecule to recruit phosphatase to dephosphorylate STAT. Second, ubiquitination may induce conformational change that would enable the association with phosphatase (Figure 1.7). Whether SLIM can also ubiquitinate other STATs remain to be investigated.

A growing body of evidence suggests that ubiquitin-mediated proteosomal degradation plays an important role in the regulation of cytokine signalling, such as JAK-STAT pathway (Figure 1.7).

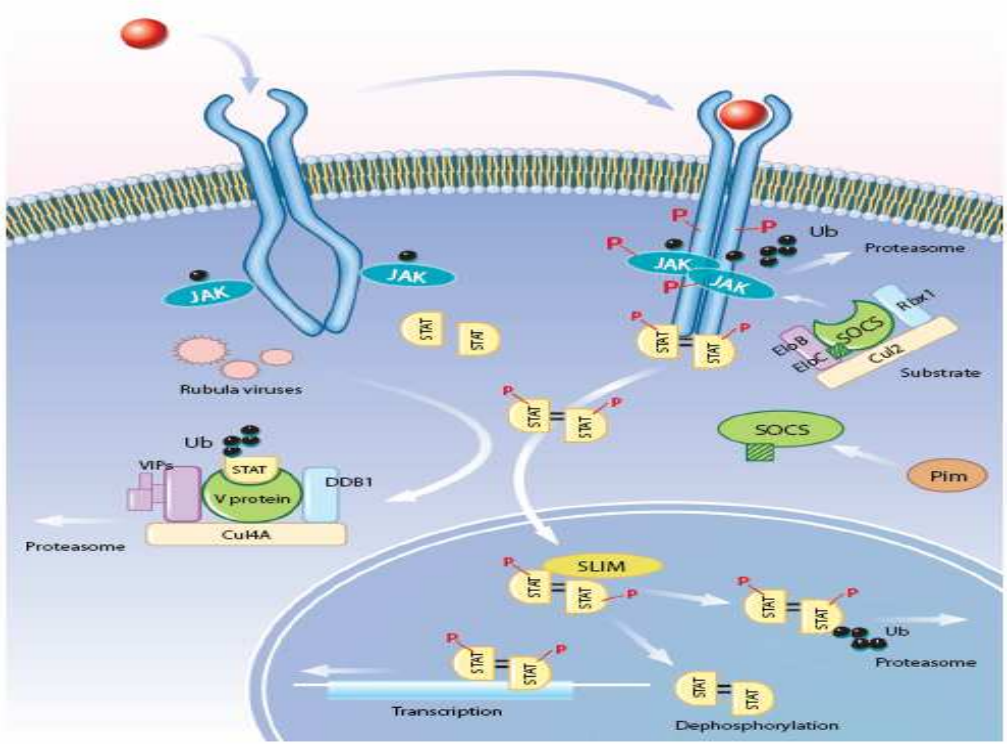


Figure 1.7 Schematic model of JAK/STAT regulation through the ubiquitin-proteasome pathway.

Ligand binding induces receptor tyrosine phosphorylation by tyrosine kinases of the JAK family, producing a docking site for the STAT family of transcription factors. Phosphorylated STAT dimers translocate to the nucleus to initiate the transcription of target genes. At the receptor complex, activated JAKs interact with SOCS proteins and become polyubiquitinated and degraded in a SOCS box–dependent manner mediated by the elongin B/C (EloB and EloC)–Cul2–Rbx1 E3 ligase complex. Rubulaviruses can target STATs for ubiquitin-mediated proteasomal degradation using a VDC E3 ligase complex that requires the participation of V protein, VIPs, DDB1, and Cul4A. In the nucleus, STAT interaction with SLIM E3 ligase can result in STAT ubiquitination and degradation or dephosphorylation (Ungureanu and Silvennoinen; 2005).

1.8 Aims and objectives of research

Immune cell trafficking between tissues compartments is regulated in part by ECs surface ARs. Endothelial cells have multiple roles in inflammation and innate

immunity. Indeed, the first events recognised as inflammation are mediated solely or largely by endothelial cells. In response to inflammatory mediators, endothelial cells express adhesion molecules, which are responsible for the recruitment of leukocytes to inflamed sites. Endothelial cells synthesise and release mediators, such as platelet activating factor, IL-8 and IL-6, which have a direct role in the inflammatory process by conducting the movement of leukocytes between tissue compartments. Functional expression of A_{2A} and A_{2B}ARs on various types of vascular ECs is well documented (Fredholm *et al.*; 2001), whereas there is little evidence for the functional expression of A_1 and A_3ARs (Montesinos *et al.*; 1997).

Moreover, it is becoming increasingly apparent that pro-inflammatory signalling pathways are subject to regulate by non-cytokine stimuli, providing an alternative control mechanism. For example, the chemokine IL-8 and bacterial-derived chemoattractant fMetLeuPhe promote the accumulation of SOCS-1 in myeloid cells and neutrophils, following activation of their cognate G-protein-coupled receptors, resulting in an inhibition of STAT3 phosphorylation in response to granulocyte colony-stimulating factor (Stevenson *et al.*; 2004). The G-protein-coupled A_{2A}AR has emerged as an important suppressor of inflammatory responses *in vivo* (Ohta and Sitkovsky; 2001; Sitkovsky *et al.*; 2004), but the mechanisms responsible for this effect remain to be fully defined. Studies in several labs and elsewhere have demonstrated that an important aspect of the A_{2A}AR's effects is its ability to inhibit NF-κB activation by multiple cell type-specific mechanisms (Majumdar and Aggarwal; 2003; Sands *et al.*; 2004). However, given its potent anti-inflammatory effects *in vivo*, this is unlikely to be the only cytokine-activated pathway affected.

Following on from these findings, I am goind to focus during my study on A_{2A}AR as a potent inhibitor of inflammatory processes. It has emerged as an important suppressor of inflammatory responses *in vivo* (Ohta and Sitkovsky; 2001; Sitkovsky *et al.*; 2004), but the mechanisms responsible for this effect remain to be fully defined. To study the effects of potentiating A_{2A}AR signalling on inhibiting inflammatory responses, we have generated a recombinant adenovirus (AV) encoding

the human A_{2A}AR gene for expression in a human umbilical vein endothelial cell (HUVEC). This facilitates highly efficient delivery of A_{2A}AR to endothelial cells *in vitro* to examine the effects of an increase in receptor signalling upon cellular response to inflammatory stimuli. The consistent anti-inflammatory role for A_{2A}AR signalling in a variety of different models of inflammation indicates a possible common mechanism of action. A considerable number of studies suggest that this inhibition may occur at the level of transcription. The aim of this project is to study the effect of A_{2A}AR-overexpression on activation of the JAK-STAT pathway in vascular ECs. Specifically, I aimed to test the effect of the A_{2A}AR-overexpression on STAT phosphorylation and expression in response to multiple cytokines (i.e sIL-6/IL-6, IFNα and leptin). The dissection of the pathways involved may ultimately lead to designing more specific and potentially useful drugs for the treatment of pro-inflammatory diseases.

At the end I would like to summarise the hypothesis of my work in two questions, which are;

Does A_{2A}AR gene transfer to HUVECs suppress sIL-6/IL-6, IFNα and leptin signalling?

Is SOCS3 induction an important mechanism or are other processes involved?

Chapter 2

Materials and Methods

2.1 Chemicals and Suppliers

All reagents were of the highest grade commercially available and obtained from the following suppliers:-

Abcam Ltd., Cambridge

Rabbit polyclonal antibody to STAT4 (cat# ab7967), Mouse mAb to anti-glyceraldehyde-3-phosphate dehydrogenase GAPDH (cat# ab8245)

Amersham Biosciences AB, Uppsala, Sweden

Glutathione-Sepharose beads^{TM} 4B,

Affinity Bioreagents, Golden, Co, USA

Anti-A_{2A}AR-specific antibody

BioRad Laboratories Ltd, Hemel Hempstead UK

Protein assay dye reagent concentration (Bradford's Reagent)

BDH Laboratories Supplies, Pooles UK

Ammonium persulphate (APS), acetic acid, isopropylalcohol, methanol, potassium chloride, potassium hydroxide, sodium chloride.

Cambrex BioScience Wokingham Ltd. Wokingham, Berkshire

Endothelial Basal Media-2^{TM} (EBM-2) and the following supplements (foetal bovine serum (FBS), hydrocortisone, fibroblast growth factor-B (hFGF-B), vascular endothelial growth factor (VEGF), insulin-like growth factor-l (IGF-l), ascorbic acid, epidermal growth factor (hEG-F), gentamicin sulphate and amphotericin-B (GA-l000) and heparin).

Human umbilical vein endothelial cells (HUVECs), SeaPlaque ® agarose, Dulbeco's modified Eagle's medium (DMAM), cell culture grade phosphate-buffered saline (PBS)

Calbiochem-Novabiochem (UK) Ltd., Nottingham

MG132, JAK inhibitor I

Cell Signalling Technology Inc., Beverly, MA USA

Anti-phospho STAT3 (Tyr705) (cat# 9138), Anti-phospho STAT1 (Tyr701) (cat# 9171), Anti-STAT3 (cat# 9132), Anti-STAT1 (cat# 9172), Anti-VEGFR2 (cat# 2479)

Duchefa Biochemie, Haarlem, Netherlands

Yeast extract, tryptone, microagar

Astra-Zeneca Pharmaceuticals Alderley Park

ZM241385

Fisher Scientific, Loughborough, Leicestershire UK

N-(2-Hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (HEPES), sodium dodecyl sulphate (SDS), ethylenediaminotetra-acetic acid (EDTA), dimethyl sulphoxide (DMSO), ethidium bromide solution, glacial acetic acid, absolute ethanol, concentrated hydrochloric acid (HCL), glycine, sodium hydroxide, Tris(hydromethyl-amino)ethane (TRIS) base, sodium carbonate, sodium hydrogen carbonate, sodium dihydrogen ortho-phosphate, di-sodium hydrogen ortho-phosphate, boric acid, chloroform, sucrose.

GIBCO BRL Life Technologies, Paisley UK

Isopropanol, OptiMEM.

Interactiva- Thermo Hybaid, Thermo Biosciences GmbH, UIm, Germany

HPLC-purified custom synthesised oligonucleotides

Inverclyde Biologicals, Strathclyde Business Park, Bellshill, UK

Schleicher and Schuell protran nitrocellulose membrane (0.2μm pore size)

Melford laboratories, Chelsworth, Ipswich, UK

Dithiothreitol (DTT), isopropyl-β-D-thiogalactopyranoside (IPTG)

New England Biolabs Inc., Beverley, MA USA

Pre-stained protein molecular weight markers (ranging from 6.5-175kDa).

Perkin-Elmer Life and Analytical Sciencesa, Monza, Italy

Enhanced chemiluminescence (ECL) reagents

Pierce, Rockford, IL, U.S.A.

Western Blot Stripping Solution, Slider-A-Lyzer ® Dialysis Cassette (0.5-3 ml capacity)

Promega, Southhampton UK

T4 DNA Ligase, Promega^{TM} Wizard plus SV DNA mini-prep kit, restriction enzymes, deoxynucleotides triphosphates (dNTPs), Ableson Maloney leukaemia virus (AMV reverse transcriptase), ribonuclease inhibitor, Taq DNA polymerase, DNA molecular size markers (ranging from 100bp-1kbp).

Qiagen, Crawley, West Sussex UK

DNA plasmid maxi-prep kit

Reidel-de Haen, Seezle, Germany

Glycerol, calcium chloride

Research Biochemicals International, Natick, MA, USA

CGS 21680

Roche Applied Science USA

FuGENE6 transfection reagent

Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

Anti-ubiquitin Antibody (PD41) (cat# D1206), Anti-STAT3 (cat# H3104)

Sigma-Aldrich Company Ltd., Poole, UK

Triton X-100, soybean trypsin inhibitor, benzamidine, IgG-free bovine serum albumin (BSA), protein A-Sepharose, sodium periodate, 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide solution, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated rabbit anti-goat IgG, thimerosal, bromophenol blue, sodium azide, agarose, deoxycholic acid sodium salt, polyethylenimine, ampicillin, paraformaldehyde, N,N,N',N'-tetramethylethylenediamine (TEMED), phenylmethylsulphonyl fluoride (PMSF), foetal bovine serum (FBS), cell culture grade trypsin EDTA, endothelial grade trypsin EDTA, penicillin, L-glutamine, bicinchonic acid salt (BCA), 3,3'5,5' -tetramethylbenzidine (TMB), human recombinant interferon-α (IFN-α), anti-FLAG® M2 monoclonal antibody F-3165, sodium fluoride, sodium potassium tartrate, deoxycholic acid (sodium salt), Trizol reagent, lysosyme, sodium orthovanadate, phenol:chloroform:isoamyl alchol (25:24:1 (v/v/v)), ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetracetic acid (EGTA), mouse anti-eNOS antibody (cat# AF400594).

Whatman International Ltd., Maidstone, Kent UK

Filter paper protran 300 mm x 3m (Conv.No# FM0535-1).

Adenoviruses (AVs) encoding Flag epitope-tagged wild-type (WT) and Tyr705→Phe-mutated murine STAT3 were generously donated by Prof. Brian Foxwell (Kennedy institute of Rheumatology, U.K.) and Prof. Keilko Yamauchi-Takihara (Osaka University Health Care Centre, Japan) and described in (Kunisada *et al.*; 1998; Williams *et al.*; 2004)

A pGEX-KG bacterial expression construct encoding a glutathione-S-transferase (GST) fusion protein containing the ubiquitin-associated (UBA) domain from *Saccharomyces cerevisiae* Dsk2p (Funakoshi *et al.*; 2002) was generously donated by Prof. Hideki Kobayashi (Kyushu University, Japan).

2.2 Cell Culture & Transfection Methods

2.2.1 Cell maintenance

All cell types were grown at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. HUVECs were maintained in endothelial basal medium supplemented with 2% (v/v) foetal bovine serum, 0.04% (v/v) hydrocortisone, 0.4% (v/v) human fibroblast growth factor-B (hFGF-B), 0.1 % (v/v) vascular endothelial growth factor (VEGF), 0.1% (v/v) insulin-like growth factor-l (IGF-l), 0.1% (v/v) ascorbic acid, 0.1% (v/v) human epidermal growth factor (hEGF), 0.1% (v/v) gentamicin sulphate and amphotericin-B (GA-1000) and 0.1% (v/v) heparin. Since HUVECs rapidly adapt in culture conditions, they were not used after passage 5 (Muller et al., 2002). For passaging, confluent cells were washed in 5 ml of PBS and then treated briefly with 3 ml endothelial grade trypsin in order to detach the cells. The trypsin was then neutralised with 10 ml of spent medium and the contents transferred to a 50 ml centrifuge tube. Cells were centrifuged for 5 min at 1000g and the supernatant was discarded. The cell pellet was gently resuspended in medium at a dilution factor suitable to establish a cell density that could be reliably counted within a standard haemocytometer, typically 1:10 dilution. Wells were then seeded at an appropriate level according to the analysis performed as indicated in the figure legends. Typically a 6-well plate would be seeded with 1 x 10^5 cells/well, ready for infection the following day with adenovirus. A minimum of $1x10^4$ cells was used to maintain the cell line in a fresh 150 cm^2 tissue culture flask to which 11 ml of fresh medium was added.

Human embryonic kidney 293 cells (HEK 293) were maintained in DMEM supplemented with 10% (v/v) FBS, 1 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Confluent monolayers were washed once in 5 ml of sterile PBS and then treated with 1 ml trypsin EDTA. Cells were then returned to the incubator for a few minutes before the flask was disrupted to dislodge the cells. Cells

were then typically diluted 1:8, 7 ml of which was used in experimental analysis and 1 ml was used to maintain the cells to which 9 ml of fresh DMEM was added.

2.2.2 Transfection with Fugene

In a sterile microfuge tube 2 μg of plasmid DNA was incubated with 200 μl of serum free medium per well of a 6 well dish and vortexed briefly. 10 μl of Fugene was added mixed gently and incubated for 15 min at room temperature. The DNA-Fugene mix was then added to each well containing 2 ml of fresh medium and left to incubate overnight. The next day, the medium was replaced with fresh growth medium. For experiments, cells were analysed 48 hr after transfection.

2.2.3 Infection of HUVECs with AVs

$1x10^5$ HUVECs were seeded in a 6 well plate and grown to 70% confluence. The cells were then infected for 24 hr at a m.o.i. of 25 pfu/cell with recombinant AVs. The next day, the medium was replaced with fresh growth medium. The following day, confluent HUVECs in six-well plates were treated as described in the figures prior to washing in ice cold PBS and solubilised by 50 μl/well detergent lysis buffer (Section 2.5.1).

2.3 Molecular Biology

2.3.1 Preparation of antibiotic agar plates

LB agar (1 % (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1 % (w/v) sodium chloride, 7% (w/v) agar) was prepared, autoclaved and allowed to cool before addition of ampicillin at the final concentrations 50 μg/ml. The liquid LB agar was then poured into 90 mm-diameter Petri dishes, allowed to solidify and then allowed to sweat overnight at room temperature to get rid of excess moisture. Plates were then stored at 4°c until required for a maximum period of 2 weeks.

2.3.2 Preparation of competent BL21 *E.coli*

An overnight culture of BL21 *E. coli* was grown in 3 ml of LB broth containing 50 μg/ml tetracycline. The following day, this was used to inoculate 250 ml of LB broth, which was then grown with aeration in a 37°C shaking incubator at 200 rpm, until the growth rate reached log phase as determined by the culture reaching an optical density at 600 nm of 0.35-0.375. Bacteria were transferred to two chilled 250 ml sterile centrifuge tubes and left for one hour on ice. Bacteria were then sedimented by centrifugation at 3,500 g for 20 min at 4°C and the supernatant discarded. The bacterial pellet was then washed and resuspended in 62.5 ml of ice-cold 0.1M magnesium chloride. Following another 20 min centrifugation at 3,500g, the bacteria were resuspended in 62.5ml of ice-cold 15% (v/v) glycerol with 0.1M calcium chloride. 250 μl of bacteria were aliquoted into sterile microfuge tubes in a ice/methanol bath to induce rapid freezing, and stored at -80 ^{o}C until required.

2.3.3 Transformation of competent BL21 *E.coli*

Approximately 30-50 ng of plasmid DNA was added to a plastic 13 ml Falcon round-bottom tube on ice. Once thawed 80 μl/tube of competent *E.coli* was immediately added and the mix incubated on ice for 30 min. The tubes were incubated for 2 min at 42°C before tube placed on ice and 1 ml of LB per tube was then added. Tubes were then shaken at 37°C for 30 min. The transformation mix was then plated out onto LB agar plates containing the appropriate selection antibiotic and incubated overnight at 37°C.

2.3.4 Preparation of plasmid DNA

Transformed colonies picked from agar plates using sterile pipette tips, were used to inoculate 10 ml of LB (1% (w/v) bactrotryptone, 0.5 % (w/v) yeast extract, 1% (w/v) sodium chloride broth supplemented with the appropriate selection antibiotic (50μg/ml ampicillin), and placed in the shaking incubator at 37°C overnight. Plasmid DNA was then isolated using the Promega ^{TM} Wizard plus SV miniprep purification

system as per the manufacturer's instructions. For larger quantities of plasmid DNA, the initial 10 ml culture was then used to inoculate a 500 ml culture containing the appropriate antibiotic and grown overnight at 37^{o}C with shaking. Plasmid DNA was isolated using the Qiagen Maxi Kit system as directed by the manufacturer's instructions. The concentration of double stranded DNA obtained was calculated based on the assumption that 1 absorbance unit (A_{260}) is equivalent to 50 μg/ml of double stranded DNA. An absorbance ratio (A_{260}/A_{280}) greater than or equal to 1.6 indicated good quality DNA.

2.3.5 Digestion of plasmid DNA

1-2 μg of purified plasmid DNA was digested in a sterile microfuge tube containing the appropriate enzyme buffer and 2-4 units of enzyme as per the manufacturer's instructions. In certain cases it was necessary, due to incompatible buffers, to purify the linearised plasmid DNA from the first digestion, before digestion with the second enzyme and buffer. This was achieved by phenol extraction and ethanol precipitation. Briefly 1/10 volume of 3M sodium acetate and 3 volumes of ice-cold absolute ethanol were added to the sample. Following 30 min incubation on ice, samples were centrifuged at 4°C for 30 min at 13,000g and the supernatant removed. The DNA pellet was washed 3x in 50 μl of 70% (v/v) ethanol, centrifuged at 4°C for 5 min at 13,000g and allowed to air-dry. It was then resuspended in 15 μl of the second enzyme buffer before the addition of 2-4 units of the second enzyme as per the manufacturer instructions. Restriction fragments were typically resolved on a 1 % (w/v) agarose gel containing 2.5 μg/ml ethidium bromide run at 75 mV for 20-30 min in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, and 0.1 % (v/v) glacial acetic acid).

2.3.6 Preparation of RNA

A 6-well plate was seeded with HUVECs at density of 1x10^{5} and was treated as described in the figure legend. Total RNA from HUVECs was isolated using Tri-

Reagent (sigma-Aldrich, Irvine, U.K.). Briefly, cells were washed 3 times in ice cold PBS and solubilised by scraping into 0.5 ml of Tri- reagent. 0.2 ml of chloroform was added and the samples were thoroughly mixed and incubated at room temperature for 5 min. Samples were then centrifuged at 13000g in a bench centrifuge for 5 min. The upper colourless layer which contains the RNA was transferred to a new RNASe tube, to which 1 ml of absolute ethanol was added. After spinning the samples at 4 ^{o}C for 30 min at full speed. The supernatant was discarded and the pellet was washed once in 1 ml of 70% (v/v) ethanol, then centrifuged for 5 min and the supernatant was discarded. The pellet was air-dried by putting it on a heater at 37 ^{o}C. Once dry, the pellet was resuspended in 15 µl of RNAase free water. The concentration of RNA obtained was calculated based on the assumption that 1 absorbance unit (A_{260}) is equivalent to 40 µg/ml of RNA.

2.3.7 Reverse transcription-PCR (RT-PCR)

For making cDNA from RNA (Section 2.3.6) 1 µg RNA was mixed with 90 ng of Random hexanucletide primers in total volume of 15 µl. Samples were heated at 70 ^{o}C for 3 minutes and then immediately placed on ice to cool. A mixture of 5x reverse transcriptase buffer (supplied with kit) and dNTPs was made and added to each reaction to give final concentrations of 50 mM Tris-HCl (pH 8.3 at 25 ^{o}C), 40 mM KCl, 8.75 mM $MgCl_2$, 10 mM DTT, 0.1 mg/ml acetylated BSA and 1mM of each dNTP. 20U of the Ribonuclease inhibitor and 5U AMV reverse transcriptase were then added, the samples mixed by pipetting and incubated at 42 ^{o}C for 3 hrs. The cDNA generated was stored at -20 ^{o}C until PCR analysis was performed.

2.3.8 PCR of prepared cDNA

PCR was carried out using GoTaq® Flexi DNA polymerase kit. Prior to this, PCR conditions were optimised to ensure that primers caused amplification of only one product of correct size (human SLIM/Mystique amplicon size of 900bp and human

GAPDH amplicon size of 150 bp). The kit provides 5x Green or Colorless GoTaq® Flexi Buffer, 25 mM $MgCl_2$ and . GoTaq® Flexi DNA polymerase

PCR was performed in a sterile nuclease-free microcentrifuge tube, by combine, the following components on ice, 17.5 µl Green Flexi Buffer,7 µl $MgCl_2$ solution (1mM for SLIM and 10 µl of 1.5 mM for GAPDH), PCR nucleotide Mix 2.8 mM each dNTP, primers for SLIM are reverse (5´-CTCAGGCCCGAGAG-3`) and forward (5´-GTATGGCGTTGACG-3`) while GAPDH are reverse GAPDH1 (5´-GAAGATGGTGATGGGATTTC-3`) and forward GAPDH2 (5´-GAAGATGGTGATGGGATTTC-3`) for GAPDH. GoTaq DNA polymerase 1.24µl and 1µl of template DNA were mixed in a final reaction volume of 25µl.

The thermal cycling conditions for the SLIM/Mystique PCR amplification were: initial denaturation at 95 ^{o}C for 2 min, followed by 30 cycles of denaturation at 95 ^{o}C for 1 min , and annealing at 58 ^{o}C for 1min followed by an extension at 72 ^{o}C for 1 min/kbp. A final extension at 72 ^{o}C has perform for 5 min.

The conditions for GAPDH were: initial denaturation at 94 for 2 min, followed by 26 cycles of denaturation at 95 ^{o}C for 1 min, and annealing at 52 ^{o}C 1min, followed by extension at 72 ^{o}C for 1 min/kbp. A final extension at 72 ^{o}C has performed for 5 min. After that products were then separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

2.3.9 Purification of adenoviral vectors

The generation and purification of plaque-purified adenoviruses (AV) encoding *myc* epitope-tagged human A_{2A}AR and GFP made by (Sands *et al.*; 2004). The presence of the gene encoding GFP in a separate open reading frame allowed us to monitor viral expression by fluorescence microscopy. 8 days post-infection low passage HEK293, cells were scraped and collected in a sterile centrifuge tube and pelleted by a 5 min centrifugation step at 400g at room temperature. The supernatant was

discarded and the pellet was washed in 1 ml sterile PBS. Cells were then disrupted by four freeze/thaw cycles in a dry ice/methanol bath and the pellet resuspended in 10 ml of sterile PBS. This was then used to infect two T-150 flasks of 50-60% confluent HEK 293 cells, grown in medium containing 2% (v/v) FBS. Virus particles from these cells were then prepared 3 days later, and used to infect a larger scale culture of 20 x T-150 flasks of HEK 293 cells. From this large scale preparation, virus was isolated as per the method of (Nicklin *et al.*; 2001). Briefly, dislodged cells were pooled and pelleted by a brief centrifugation at 250g for 10 min at room temp. The supernatant was then discarded and the pellet resuspended in 10 ml of sterile PBS. The pellet was then washed four times with 1 ml of sterile PBS and the supernatant discarded. This was then subjected to four freeze/thaw cycles in order to lyse the cells and release the virus particles. The preparation was then added to a discontinuous caesium chloride gradient set up in a 13ml sterile ultracentrifuge tube, and was subjected to centrifugation at 100,000g for 90 min at 8°C with zero deceleration. The opaque adenovirus band was then isolated by syringe extraction and added into a Slide-A-Lyser that allowed efficient overnight dialysis at 4°C in lL of dialysis buffer (15 mM sodium chloride, 51.2 μM Tris (pH 7.5), 10 μM EDTA) which was changed 3 times. Virus samples were removed from the Slide-A-Lyser and diluted in a 1:1 (v/v) ratio with sterile virus storage buffer (10 mM Tris-HC1, pH 8, 100 mM sodium chloride, 0.1 % (w/v) BSA, 10% (v/v) glycerol) and stored at -80°C in 30 μl aliquots.

Once isolated, the virus was subject to titration by the end-point dilution method (Nicklin and Baker, 2001) using GFP as a marker for positive colonies. 1 x 10^4 HEK 293 cells/well was subcultured into 8x10 wells of a 96-well plate in order to reach approximately 50-60% confluency after 24 hr growth. 0.1 ml of virus was then used to infect the wells over a range of serial dilutions (10^{-2}-10^{-11}). This was incubated for 18 hr before the virus was removed and the medium replaced. The medium was replaced every 3 days up to 10 days post-infection until the well showed evidence of viral infection as assessed by GFP expression. After 10 days the number of virus positive wells was counted for each concentration of virus and the virus titre was.

2.3.10 Preparation of GST-UBA Sepharose beads

10 ml of LB broth supplemented with 50 μg/ml ampicillin was inoculated from a glycerol stock of BL21 *E.*coli transformed with Dsk2pUBA was then grown overnight, shaking at 37 ^{o}C. This starter culture was then used to inoculate 400ml LB containing 50μg/ml ampicillin was grown shaking at 200 rpm for 5 hours (or until OD_{600} = 0.3 or greater) at 37 ^{o}C. Fusion protein expression was then induced by addition of IPTG to a final concentration of 1mM, and then grown for 4 hours at 37 ^{o}C with shaking at 200 rpm. The bacteria were then harvested by centrifugation at 6700g for 15 min, and the supernatant discarded. The cells were resuspended in 20ml lysis buffer (50mM sodium HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA and 1% (v/v) Triton-X-100) and incubated at room temperature for 30 min. The samples were then probe sonicated on ice 3 times for 30 second to ensure efficient cell lysis and then centrifuged at 27000g for 30 mins to pellet insoluble material. The cleared lysate was then added to 0.6 ml 50 % (v/v) glutathione-Sepharose bead suspension and placed on a rotating wheel at 4 ^{o}C for 1 hour to allow the fusion protein to bind. The beads were then washed three times with 10 ml PBS and, following the final wash, resuspended in 50% (v/v) glycerol in PBS supplemented with protease inhibitors (0.1 mM PMSF, 10 μg/μl soybean trypsin inhibitor and 10 μg/μl benzamidine) for storage at -20 ^{o}C. The same procedure was utilised for the preparation of GST-immobilised Sepharose beads which were used as a negative control.

2.3.11 SPAGE assays for quantitation of immobilised GST fusion proteins.

20 μl of prepared bead suspension was centrifuged at 5000g for 20 sec at 4 ^{o}C and the supernatant was removed with a 1 ml syringe. The protein was eluted from the beads by addition of 20 μl of 2% (w/v) SDS sample buffer and incubated at 37 ^{o}C for 30 min. The sample was briefly centrifuged at room temperature and the protein containing supernatant was removed via a Hamilton syringe into a fresh microfuge

tube. Known amounts of BSA ranging from 0.2-2 μg and 5μl and 10μl of eluted proteins were analysed by SDS PAGE using a 10% (w/v) resolving gel. The gel was then stained for 1 hr at RT with Coomassie Brilliant Blue (3mM Coomassie Brilliant Blue G, 45% (v/v) methanol, 10% (v/v) acetic acid). Following destaining overnight, the gel was then scanned and the density of the BSA bands were used to generate a standard curve from which the concentration of protein immobilised on the GST-Sepharose beads could be determined (Figure 2.1). The same method used for GST

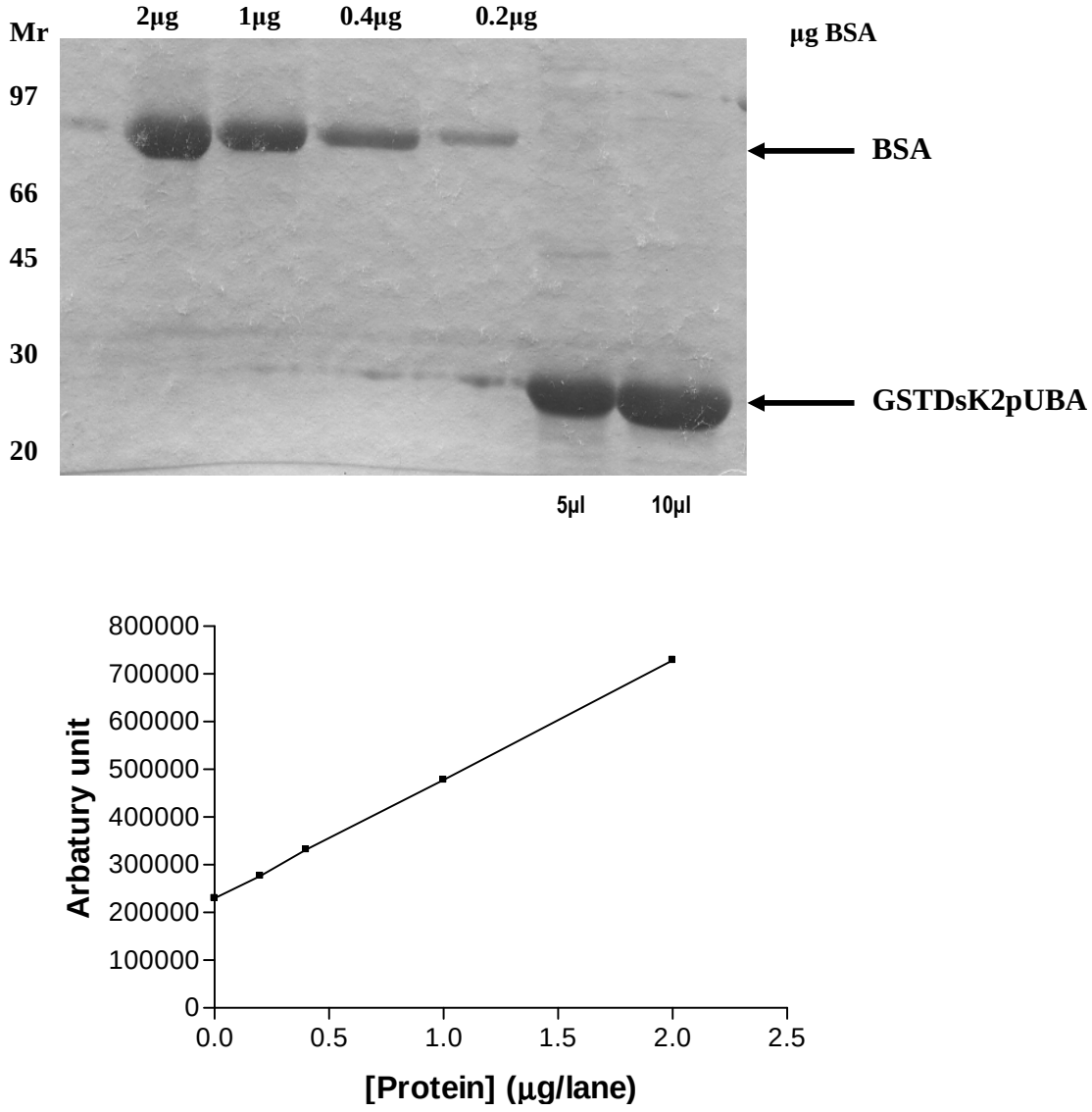


Figure 2.1 SDS-PAGE protein assay for immobilised GST fusion proteins on beads

Known amounts of BSA ranging from 0.2-2 µg and 5µl and 10µl of eluted proteins were analysed by SDS PAGE. The gel was stained for 1 hr at RT with Coomassie Brilliant Blue (3mM Coomassie Brilliant Blue G, 45% (v/v) methanol, 10% (v/v) acetic acid). The gel was destained overnight, scanned and the density of BSA bands was used to generate a standard curve. The BSA standard curve was then used to

estimate the concentration of protein immobilised on the beads. The same method was used for GST.

2.4 Laboratory techniques

2.4.1 Cell lysis

Confluent HUVECs in six-well plates were treated as described in the figures prior to washing in ice cold PBS and solubilised by 50 µl/well detergent lysis buffer (50mM sodium HEPES, pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium phosphate , 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.1 mM phenylmethylsulphonyl fluoride, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml benzamidine and EDTA-free complete protease inhibitor mix). Samples were then scraped pipeting to microfuge tube and followed by brief vortexing, insoluble material was removed by microcentrifugation at 5000g for 5 min at 4 ^{o}C and the supernatant assayed for protein concentration using a bicinchonnic acid assay described in (Section 2.5.2).

2.4.2 Discontinuous SDS-PAGE and Immunoblotting

Samples were equalised for protein amounts (typically 10-20 µg/sample) and following the addition of an equal of samples buffers were fractionated by discontinuous SDS-PAGE using a 6 cm 10% (w/v) polyacrylamide resolving gel (10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4 M Tris (pH 8.8), 0.1 % (w/v) SDS, 3% (v/v) glycerol, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and a 2 cm 3% (w/v) stacking gel (3% (v/v) acrylamide, 0.1% (v/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.001 % (v/v) TEMED). Pre-stained protein markers (Invitrogen Rainbow Markers, range 6.5-175kDa) in sample buffer (50 mM Tris (pH 6.8), 10% (v/v) glycerol, 12% (w/v) SDS, 0.0001 % (w/v) of bromophenol blue, 1 mM DTT) were also used in order to determine protein molecular mass. Electrophoresis was carried out using Biorad Mini-Protean II or III gel electrophoresis systems in running buffer (27.4 mM Tris, 0.19M glycine, 0.1 % (w/v) SDS) at 150 V until the

bromophenol blue dye front reached the bottom of the gel. Proteins were then transferred electrophoretically onto a nitrocellulose membrane at 400 mA for 45 min in transfer buffer (24.7 mM Tris, 0.19 M glycine in 20% (v/v) methanol). Following transfer to nitrocellulose, membranes were blocked for one hour at room temperature in blocking solution (5% (w/v) skimmed milk in Tris-buffered saline (TBS) containing 0.1 % (v/v) Tween-20 (TBST) or 5% (w/v) skimmed milk in phosphate-buffered saline (PBS), 0.2% (v/v) Triton X-100 in PBS. Antibody, diluted in Blotto as indicated in the Figure Legends. This was incubated in the cold room overnight on a rotating platform. The membranes were rinsed briefly in PBS before being washed three times in Blotto for 10 min. Membranes were then washed again briefly in PBS before being transferred to a bag containing 2 ml of the appropriate secondary antibody conjugated to HRP in High-Detergent Blotto (10% (v/v) Blotto in PBS supplemented with 1.02% (v/v) Triton X-100 and 0.1 % (w/v) SDS). This was then placed on a rotating platform at room temperature and incubated for 1 hr. The membrane was then washed three times for 10 min in Blotto and then washed a further twice for 10 minutes in PBS. Membranes were then exposed to an enhanced chemiluminescent procedure in which HRP-specific oxidative degeneration of luminal causes emission of light at 428 nm which is detected by Kodak XOMAT Blue X-ray film (see Table 2.1).

2.4.3 Protein concentration determination using the bicinchonic acid (BCA) protein assay

Duplicate 10µl samples of known BSA standards in the range 0-2 mg/ml and unknown protein samples were added to a 96-well plate. 0.2 ml BCA solution (1 % (w/v) 4,4 dicarboxy-2,2 biquinoline disodium salt, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium bicarbonate pH 11.25, 0.08% (w/v) copper (II) sulphate) was then added to each well. Protein concentration-specific reduction of Cu^{2+} to Cu^{1+} allows the bicinchoninic acid sodium salt to bind the Cu^{1+} ion forming an intense purple colour allowing measurement of the absorbance at 492 nm using a plate reader. Colour was

There fore allowed to develop at room temperature for 30 min and the absorbance of the standards was used to determine a straight line from which unknown protein concentrations could be calculated (Smith et al, 1985).

Table 2.1 Antibody Incubation Conditions

PROTEIN	INCUBATION CONDITIONS		BLOTTO	
	1ºAB	**2ºAB**	**1ºAB**	**2ºAB**
P-STAT1	P-STAT1 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
STAT1	STAT1 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) skimmed milk PBS TWEEN	5% (W/V) skimmed milk PBS TWEEN
P-STAT3	P-STAT3 1:1000 Overnight, cold room	Anti-mouse HRP 1:1000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
STAT3	STAT3 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) skimmed milk PBS TWEEN	5% (W/V) skimmed milk PBS TWEEN

P-STAT4	P-STAT4 1:1000 Overnight, cold room	Anti-mouse HRP 1:1000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
Ub	Ub 1:1000 Overnight, cold room	Anti-mouse HRP 1:1000 1hr, room temp	5% (W/V) skimmed milk PBS TWEEN	5% (W/V) skimmed milk PBS TWEEN
GAPDH	GAPDH 1:20000 1hr, room temp	Anti-mouse HRP 1:5000 1hr, room temp	5% (W/V) skimmed milk PBS TWEEN	5% (W/V) skimmed milk PBS TWEEN
VEGFR2	VEGFR2 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
eNOS	eNOS 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:2000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
SLIM	SLIM 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
Flag	Flag 1:1000 Overnight, cold room	Anti-mouse HRP 1:1000 1hr, room temp	5%(W/V) skimmed milkPBS TWEEN	5%(W/V) skimmed milkPBS TWEEN

P-JAK1	P-JAK1 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
P-JAK2	P-JAK2 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) BSA TBST	5% (W/V) BSA TBST
JAK1	JAK1 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) skimmed milk PBS TWEEN	5% (W/V) skimmed milk PBS TWEEN
JAK2	JAK2 1:1000 Overnight, cold room	Anti-Rabbit HRP 1:1000 1hr, room temp	5% (W/V) skimmed milk PBS TWEEN	5% (W/V) skimmed milk PBS TWEEN
GFP	GFP 1:10000 Overnight, cold room	Anti-Goat or Anti-Sheep 1:30000 1hr, room temp	5% (W/V) skimmed milk TBS TWEEN	5% (W/V) skimmed milk TBS TWEEN

2.4.4 GST-Dsk2pUBA pull-down assays

Recombinant GST and GST-Dsk2pUBA were purified from transformed *E. coli* BL21 (DE3) cultures using glutathione-Sepharose beads following induction of fusion protein expression with IPTG as described in (Section 2.3.10). For pull-down

assay, confluent HUVECs in six-well dishes were treated as described in the results prior to termination of the reactions by addition of ice cold PBS. All subsequent procedures were performed at 4^{o}C unless indicated otherwise. Cells were solubilised by scraping into 0.25 ml/well pull-down lysis buffer (50mm sodium HEPES, pH 7.5, 150 mM sodium chloride, 5mM EDTA, 1mM sodium vanadate, 6 μM MG132, 1 mM N-ethylmaleimide, 1% (v/v) Triton X-100, 0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine and EDTA-free complete protease inhibitor mix) and incubated on ice for 30 min. Following centrifugation at 5000g for 5 min at 4 ^{o}C to pellet insoluble material, samples were equalised for volume and protein content (typically 50 μg in 0.2 ml) prior to the addition of either GST or GST-Dsk2pUBA (5μg/sample) immobilised to glutathione-Sepharose beads and incubated with rotation for 1 hr. Beads were recovered by brief centrifugation and washed five times with 1 ml lysis buffer prior to the elution of bound protein by the addition of 50μl electrophoresis sample buffer containing 12% (w/v) SDS and incubation at 60 ^{o}C for 15 min. Sample were then fractionated by SDS-PAGE and transferred to nitrocellulose for immunoblotting.

To remove polyubiquitin chains from captured proteins prior to SDS-PAGE, beads were washed twice with 1ml deconjugation buffer (50 mM sodium HEPES, pH7.5, 1mM dithiothretol) after the lysis buffer wash steps and then resuspended in 50μl deconjugation buffer supplemented with 0.2 μM recombinant human isopeptidease T/UBP5 and incubated at 37 ^{o}C for 1 hr. Reactions were terminated by brief centrifugation and the supernatant containing deconjugated proteins was removed for analysis by SDS-PAGE and immunoblotting following the addition of electrophoresis sample buffer.

2.4.5 Immunoprecipitation

Confluent HUVECs in six well dishes were pre-incubated with 6 μM MG132 for 30 min prior to treatment with or without sIL-6Rα/IL-6 as described in the figure

legends prior to termination of the incubation by placing dishes on ice and washing cell monolayers three times with ice-cold PBS. Cells were solubilised by scraping into 0.1 ml denaturing lysis buffer (50 mM sodium HEPES, pH 7.5, 100 mM sodium chloride, 1 mM N-ethylmaleimide, 2% (w/v) SDS, 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg /ml benzamidine and EDTA-free complete protease inhibitor mix). The samples were then incubated at 95 ^{o}C for 5 min followed by probe sonication on ice 3 times for 30 second. After the addition of 0.9 ml lysis buffer containing sufficient Triton X-100 and sodium deoxycholate to give final concentrations of 1% (w/v) and 0.5% (w/v) respectively, insoluble material was removed by centrifugation at 5000g for 5 min at 4 ^{o}C and soluble fractions equalised for protein content volume prior to incubation for 1 hr at 4 ^{o}C with rotation with 25 μl packed volume of protein A-Sepharose beads in the presence of 0.2% (w/v) IgG-free BSA. Anti-STAT3 antibody (2μg/sample) was then added and the incubation continued for a further 1 hr. Immune complexes were isolated by brief centrifugation at 5000g for 5 min at 4 ^{o}C and washed three times with 1 ml detergent lysis buffer prior to elution of precipitated proteins by the addition of 40 μl electrophoresis sample buffer containing 12% (w/v) SDS and incubation at 60 ^{o}C for 15 min. Samples were then fractionated by SDS-PAGE using 7.5% (w/v) polyacrylamide resolving gels and transferred to nitrocellulose for immunoblotting.

Confluent HUVECs in six well dishes were pre-incubated with 6 μM MG132 for 30 min prior to treatment with or without sIL-6Rα/IL-6 as described in the figure legends prior to termination of the incubation by placing dishes on ice and washing cell monolayers three times with ice-cold PBS. Cells were solubilised by scraping into 0.1 ml denaturing lysis buffer (50 mM sodium HEPES, pH 7.5, 100 mM sodium chloride, 1 mM N-ethylmaleimide, 2% (w/v) SDS, 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg /ml benzamidine and EDTA-free complete protease inhibitor mix). The samples were then incubated at 95 ^{o}C for 5 min followed by probe sonication on ice 3 times for 30 second. After the addition of 0.9 ml lysis buffer containing sufficient Triton X-100 and sodium deoxycholate to give

final concentrations of 1% (w/v) and 0.5% (w/v) respectively, insoluble material was removed by centrifugation at 5000g for 5 min at 4 ^{o}C and soluble fractions equalised for protein content volume prior to incubation for 1 hr at 4 ^{o}C with rotation with Recombinant Flag-tagged STAT3 was immunoprecipitated by the addition of 20 µl packed volume of anti-Flag M2-Sepharose beads and incubation with rotation for 1 hr at 4 ^{o}C. Immune complexes were isolated by brief centrifugation at 5000 xg for 5 min at 4 ^{o}C and washed three times with 1 ml detergent lysis buffer prior to elution of precipitated proteins by the addition of 40 µl electrophoresis sample buffer containing 12% (w/v) SDS and incubation at 60 ^{o}C for 15 min. Samples were then fractionated by SDS-PAGE using 7.5% (w/v) polyacrylamide resolving gels and transferred to nitrocellulose for immunoblotting.

2.4.6 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl 2H-tetrazolium bromide (MTT) assay of cell viability

HUVECs were seeded in a 24 well plate and grown to 70% confluence. The cells were then infected for 48 hr with recombinant AVs. On the next day, the medium was replaced with fresh growth medium. The following day, cells were treated with sIL-6Rα/IL-6 for 3hr or 0.1 (w/v) sodium azide for 16 hr. Then 20 µl of 12 µM MTT (3-[4,5-dimethylthioazol-2-yl] was added to each well for 2 hrs. After that the medium in use was discarded and 200 µl of dimethyl sulfoxide (DMSO) was used to solubilize samples. 150 µl from each well was then added to 96 well plates and the formazan product was determined by measuring the absorbance at 550 nm.

2.4.7 Statistical Analysis

Statistical analysis was carried out using the Student t-test using GraphPad Instat 3 as indicated in the Figure Legends. Significance was assessed as $p<0.05$.

Chapter3

Effect of A_{2A}AR Expression on Cytokine Activation of the JAK-STAT pathway in vascular ECs

3.1 Introduction

Endothelial cells were once viewed as relatively inert cells lining the vasculature. They are now recognized as active and responsive regulators of coagulation, platelet adhesion, fluid homeostasis, wound healing, leukocyte extravasation and vascular tone. Endothelial cells play a key role in the host response to infectious agents by regulating leukocyte trafficking, producing inflammatory cytokines and presenting antigen in association with major histocompatibility class II (MHC II) molecules (Cid *et al.*; 1998) . A number of infectious induce pathology by interacting with endothelial cells. Infection of endothelial cells can promote thrombosis, vascular leakage, and increased adherence and emigration of leukocytes. Furthermore, activation of a systemic inflammatory response, in the absence of direct endothelial cell infection, can also lead to endothelial cell dysfunction. The endothelial dysfunction occurs under activation conditions, with the acquisation of many new functional, inflammatory and immune properties. A consequence of this is to display many different transcription profiles. In addition, endothelial cells selectively control vascular permeability, which is important in many pathophysiological processes (Mader; 1996).

Endogenous adenosine is a potent regulator of inflammation. It has been reported that adenosine accumulation underlines the anti-inflammatory action of anti-rheumatic drugs such as methotrexate (Montesinos *et al.*; 2000). Monocytes and macrophages synthesise and release into their environment a variety of cytokines and other proteins that play a central role in the development of acute and chronic inflammation. There is evidence suggesting a regulatory connection between adenosine and its receptors and inflammatory cytokines. In human and murine monocytes/macrophages, the activation of adenosine receptors, particularly A_{2A}AR, by adenosine or it analogues, modulates the production of inflammatory cytokines including TNF-α, IL-10, and IL-12 (Hasko *et al.*; 2000). IL-12, a pro-inflammatory cytokine and a central inducer of

Th1 responses and cell-mediated immunity, is suppressed by adenosine and its analogues (Hasko *et al.*; 2000; Link *et al.*; 2000) .

In human peripheral blood mononuclear cells, treatment with cAMP-elevating agents, such as the PDE4 inhibitor rolipram, causes complete inhibition of TNF-α production and an increase in IL-10 synthesis (Eisenhut *et al.*; 1993). In the same way, cAMP agonists inhibit endotoxin-induced IL-6 expression in monocytes or macrophages differentiated in cell culture (Eigler *et al.*; 1998). Identification of A_{2A}AR as natural brakes of inflammation has provided a useful framework for understanding how tissues regulate inflammation. Thus, targeting the endogenous inflammatory pathways such as JAK-STAT signalling pathway by the A_{2A}AR might be a useful strategy in the clinical management of inflammation. The aim of this chapter was to determine what effect adenoviral gene transfer of the *myc*-His-tagged human A_{2A}AR would have on the inflammatory response in HUVECs, and what A_{2A}AR-stimulated molecular mechanism were involved.

A_{2A}ARs have a non-redundant role in the attenuation of inflammation and tissue damage in *vivo*. Sub-threshold doses of an inflammatory stimulus as ((Tiegs; 1997; Kaneko *et al.*; 2000) showed cause minimal tissue damage in wild-type mice were found to cause extensive tissue damage, more prolonged and higher levels of pro-inflammatory cytokines, and death of male animals deficient in the A_{2A}AR. Similar observations were made in studies of three different models of inflammation and liver damage as well as during bacterial endotoxin-induced septic shock. Previous studies suggest that A_{2A}AR plays a critical role in the physiological negative feedback mechanism for limiting and terminating both tissue-specific and systemic inflammatory responses. Accumulation of extracellular adenosine in inflamed and damaged tissues (Rudolphi *et al.*; 1992) and the immunosuppressive properties of cAMP-elevating adenosine receptors (Cronstein; 1994; Huang *et al.*; 1997; Sullivan *et al.*; 2000) indicate that A_{2A}AR signalling in immune cells is a possible natural mechanism of inhibition and/or termination of inflammation.

3.2 Results

HUVECS are a commonly used endothelial cell in studies of inflammation (Ahn *et al.*; 2003; Carman *et al.*; 2003), as they grow more easily in cell culture compared to other human endothelial cell type. It is widely accepted that a heterogeneity of endothelial function (Yang *et al.*; 1991) and phenotype exists depending on the vascular bed of origin (Page *et al.*; 1992). One of the major documented differences between endothelial cells, as far as inflammatory processes are concerned, relates to responses to cytokines. HUVEC respond to cytokines such as sIL-6Rα/IL-6 and IFNα by changinly their targe gene expression as well as allowing mononuclear cell transmigration. However, a disadvantage of using primary endothelial cells is the difficulty with which new cDNAs can be introduced. This was important as any inhibitory effect of the A_{2A}AR activation in transfected cells would be undetected if transfection efficiencies were poor. Generally standard transfection methods yield poor transfection efficiencies (2-10%) in endothelial cells (Teifel *et al.*; 1997). Therefore, in order to bypass this problem, a replication-deficient recombinant adenovirus encoding a *myc*-His-tagged human A_{2A}AR, was used to obtain higher level of A_{2A}AR infection.This facilitates highly efficient delivery of A_{2A}AR to endothelial cells in *vitro* to examine the effects of an increase in receptor signalling upon cellular responses to class I cytokines. Several experiments have been designed to investigate the effect of overexpression of A_{2A}AR in HUVECs to the same level as endogenous A_{2A}AR level during the inflammation such as in hypoxia and its ligands on the function of IL-6, IFNα and Leptin in HUVECs. Specifically, experiments tested the effects of IL-6 and IFNα on the activity of downstream signal transduction proteins (JAK-STAT pathway). Previous work undertaken in our lab has shown that A_{2A}AR-mediated inhibition of nuclear factor κB (NFκB) activation is a critical aspect of its anti-inflammatory signalling properities and that the molecular basis of this inhibition is cell type-specific (Sands *et al.*; 2004). Thus, experiments initially tested the effect of simply overexpressing the A_{2A}AR (Figure 3.1A) on cytokine stimulation of the JAK-STAT pathway in these cells. It was necessary to ensure adequate A_{2A}AR

overexpression levels were achieved via adenoviral gene transfer before utilising this binding to examine the effect of the A_{2A}AR on JAK-STAT pathway. Adenoviral gene transfer was necessary due to the passage-dependant loss of endogenous A_{2A}AR expression observed in HUVECs. Confluent monolayers of HUVECs were infected with 25pfu/cell of either AdA_{2A}AR or control virus AdGFP, which was required to achive consistent A_{2A}AR-expression. Infected cells were identified under fluorescence by the presence of green fluorescent protein GFP). (Figure 3.1B) shows A_{2A}AR gene transfer in HUVECs under phase and fluorescence light with the infected efficiency was determined to be ¬75%. GFP gene transfer was found to have an infection efficiency of ¬75% (Figure 3.1B). Moreover, immunoblot probe with anti-GFP antibody shows there is no different in GFP-expression between GFP-overexpressing cells and A_{2A}AR-overexpressing cells (Figure 3.1C).

In control GFP-overexpressing cells, treatment with a sIL-6Rα/IL-6 trans-signalling complex produced a transient increase in the phosphorylation of STAT1 on Tyr701 and STAT3 on Tyr705. Overexpression of the A_{2A}AR significantly reduced STAT phosphorylation at each time point after 15 min (Figure 3.2 and 3.3). Interestingly, while total levels of STAT1 and STAT3 were unaltered by sIL-6Rα/IL-6 treatment of GFP-overexpressing cells, a marked decreased in the amount of total STAT1 and STAT3 in A_{2A}AR-overexpressing HUVECs was detectable from as early as 30 min (Figure 3.2 and 3.3). To determine whether this effect was restricted to sIL-6Rα/IL-6, the effect of A_{2A}AR overexpression on IFNα-mediated activation of the JAK-STAT pathway was also tested. In GFP-overexpressing cells, IFNα treatment produced a more transient increase in the Tyr-phosphorylation of STAT1 and STAT3 as sIL-6Rα/IL-6. Nevertheless, A_{2A}AR overexpression inhibited phosphorylation at each time point (Figure 3.4 and 3.5). In addition, levels of total STAT1 and STAT3 were markedly reduced as early as 15 min following IFNα exposure (Figure 3.4 and 3.5). More extensive characterisation of the effect of sIL-6Rα/IL-6 and IFNα on STAT levels over time revealed that for sIL-6Rα/IL-6, STAT1 and STAT3 were reduced to almost undetectable levels after 3hrs in A_{2A}AR-overexpressing HUVECs (Figure 3.6

and 3.7) whereas, for IFNα, STAT1 and STAT3 were reduced to almost undetectable levels after 2hrs (Figure 3.8 and 3.9). In contrast, cytokine exposure of GFP-overexpressing HUVECs under the same conditions produced no detectable change in total levels of STAT1 or STAT3 in response to either cytokine, demonstrating that the down-regulation effect is specific for overexpression of the A_{2A}AR (Figure 3.2-3.9). Finally, the effect of the (A_{2A}AR-selective agonist) CGS21680 and the (A_{2A}AR antagonist) ZM241385 on cytokine stimulation of the JAK-STAT pathway in these cells was investigated. Thus, to assess the functional consequences of cAMP elevation on sIL-6Rα/IL-6. The effect of A_{2A}AR overexpression on STAT1 and STAT3 phosphorylation and down regulation could be rescued by co-incubation of sIL-6Rα/IL-6 with the A_{2A}AR-selective antagonist ZM241385 (3.10, 3.11). Conversely, co-incubation with the selective agonist CGS21680 did not further potentiate the effect of receptor expression to down regulate STAT in A_{2A}AR-overexpressing cells (Figure 3.10, 3.11).

Importantly, the cytokine-stimulated decrease in STAT phosphorylation did not simply reflect reduced HUVECs viability following A_{2A}AR overexpression. For that the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl 2H-tetrazolium bromide (MTT assay) used to demonstrate that GFP- and A_{2A}AR-overexpressing HUVECs had similar viabilities that were not affected by sIL-6Rα/IL-6 exposure at a 3 hr time point at which STAT degradation was detectable only in A_{2A}AR-overexpressing cells (Figure 3.12).

The ubiquitin-proteasome pathway plays a central role in the targeted destruction of cellular proteins, including cell cycle regulatory proteins. Because these pathways are critical for the inflammation, proliferation and survival of all cells, proteasome inhibition is a potentially attractive therapeutic target in eukaryotic cells (Section 1.6). Regulated degradation is a commonly utilised mechanism by which cellular levels of transcription factors, such as p53 (Watson and Irwin; 2006), are controlled. To determine whether STATs are targeted for degradation by the proteasome,

experiments were performed to investigate the effect of proteasome inhibition on the ability of the A_{2A}AR overexpression to prime STATs for cytokine-mediated down-regulation. Pre-incubation with the proteasome inhibitor MG132 was sufficient to abolish the effect of the A_{2A}AR overexpression on priming both STAT1 and STAT3 for down-regulation in response to sIL-6Rα/IL-6 (Figure 3.13 and 3.14). In addition, Mg132 was sufficient to abolish the effect of the A_{2A}AR overexpression on priming both STAT1 and STAT3 for down-regulation in respone to IFNα (Figure 3.15 and 3.16). Blocking of proteasomal degradation with MG132 abolished A_{2A}AR inhibition of both sIL-6Rα/IL-6 and IFNα-mediated phosphorylation of STAT1 and STAT3, suggesting that STAT degradation is the only mechanism responsible for this effect (Figure 3.17-3.20).

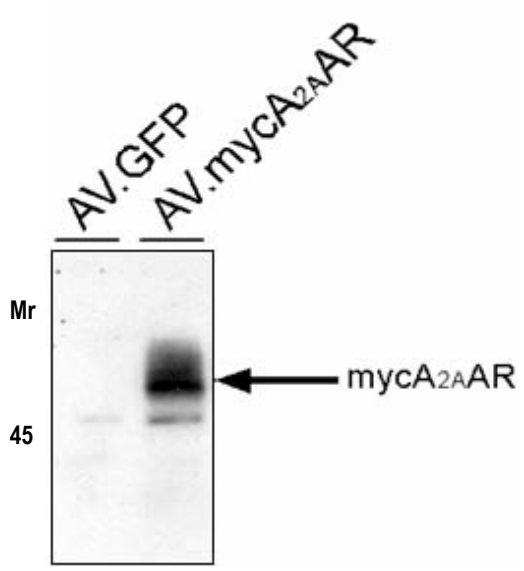
Figure 3.1 A_{2A}AR gene transfer to HUVECs

Panel A: HUVECs were infected with the indicated AVs at an MOI of 25 *pfu /cell* as indicated in the Materials and Methods prior to preparation of soluble cell extracts for SDS-PAGE and immunoblotting with anti-myc Ab 9E10 to identify recombinant A_{2A}ARs.

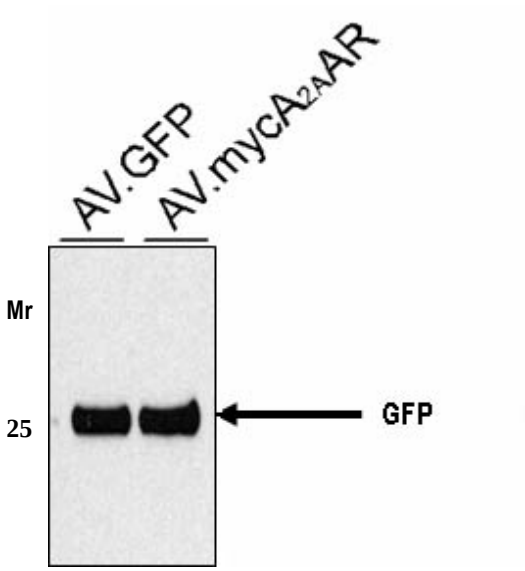
Panel B: 48hrs post-infection, HUVECS were examined for GFP via fluorescent microscopy.

Panel C: HUVECs were infected with the indicated AVs at an MOI of 25 *pfu /cell* as indicated in the Materials and Methods prior to preparation of soluble cell extracts for SDS-PAGE and immunoblotting with anti-GFP Ab to identify the GFP-expression between GFP-overexpressing cells and A_{2A}AR-overexpressing cells.

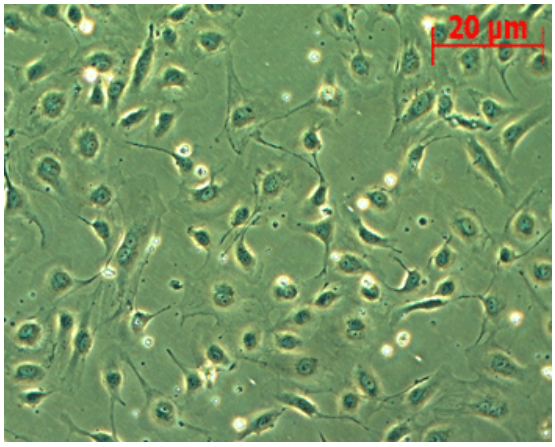
A



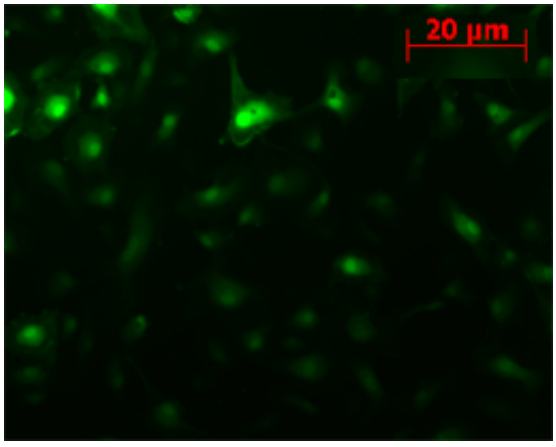
C



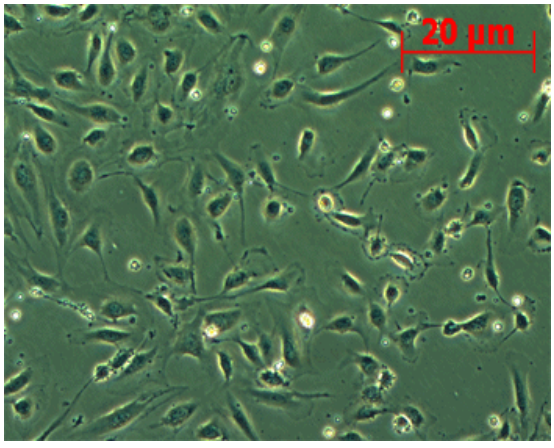
B



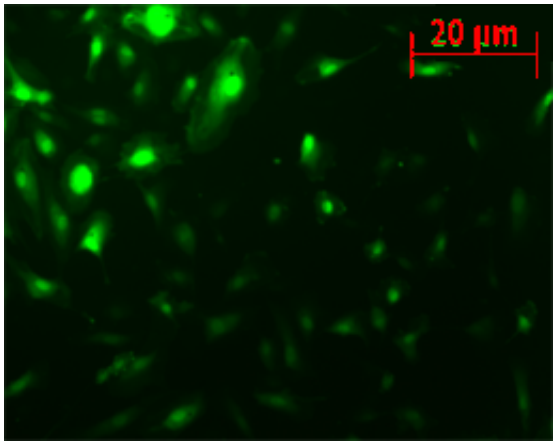
Phase microscopy



AV/GFP infected HUVECs via fluorescence



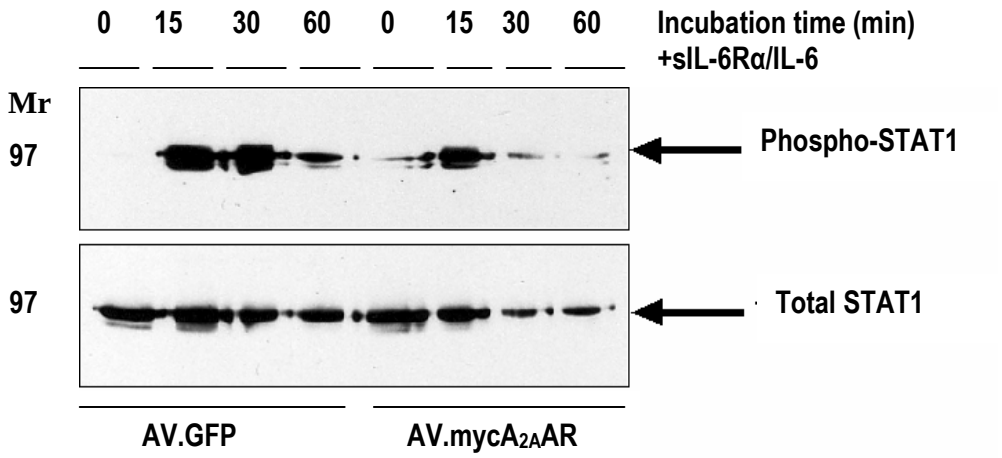
Phase microscopy

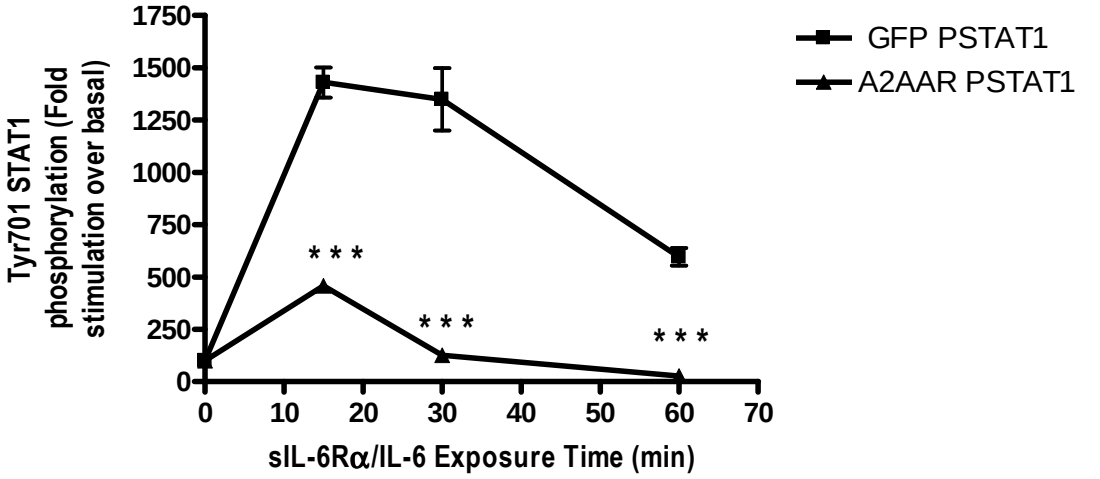


AV/A_{2A}AR infected HUVECs via fluorescence

Figure 3.2 Effect of A_{2A}AR gene transfer on STAT1 phosphorylation and expression in response to sIL6Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT1 and total STAT1 levels from three experiments is presented (***p<0.001 and *P<0.05 *versus* the response observed in AV.GFP-infected cells). Basal set at 100.


0 15 30 60 0 15 30 60
Incubation time (min)
+sIL-6Rα/IL-6
Mr
97
Phospho-STAT1
97
Total STAT1
AV.GFP
AV.mycA2AAR


GFP PSTAT1
A2AAR PSTAT1
Tyr701 STAT1 phosphorylation (Fold stimulation over basal)
1750
1500
1250
1000
750
500
250
0
* * *
* * *
* * *
0 10 20 30 40 50 60 70
sIL-6Rα/IL-6 Exposure Time (min)

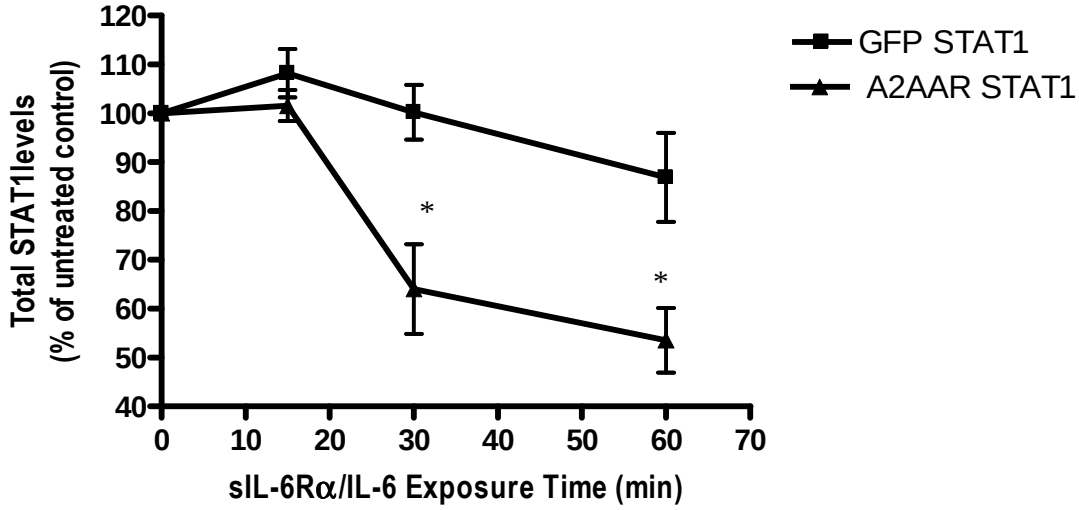
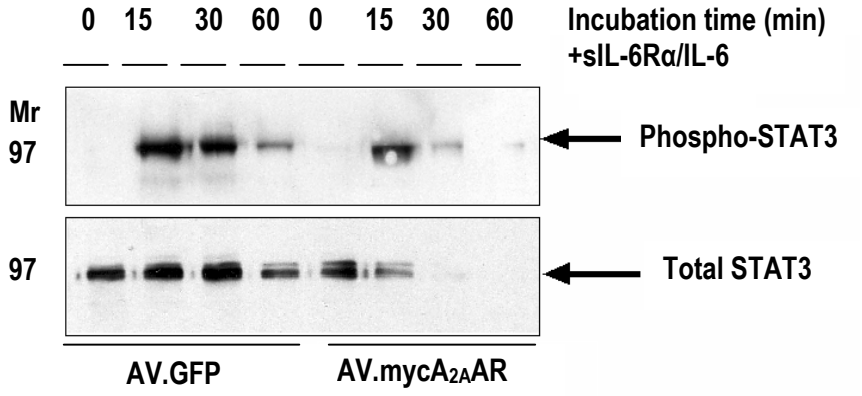
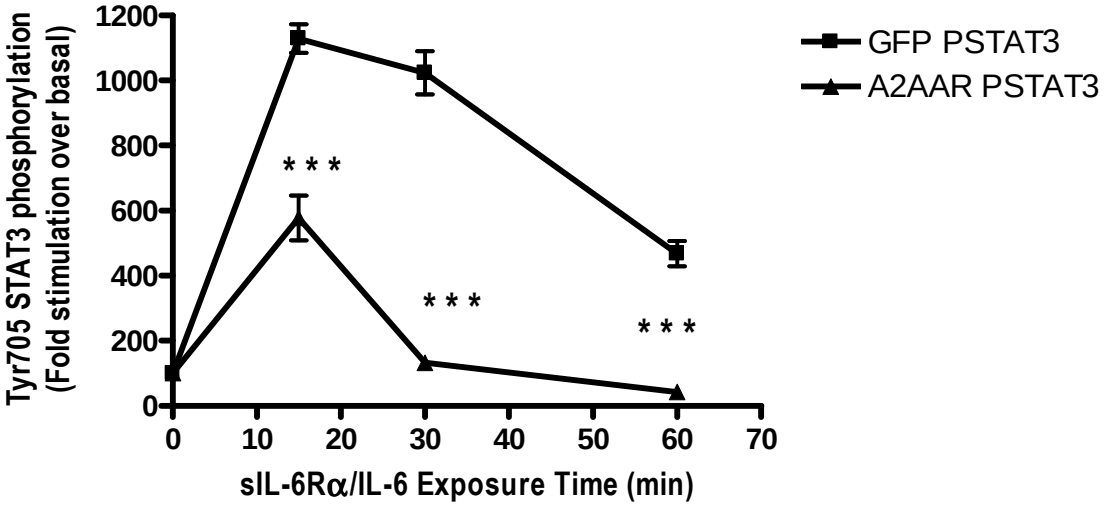

GFP STAT1
A2AAR STAT1
Total STAT1levels (% of untreated control)
120
110
100
90
80
70
60
50
40
*
*
0 10 20 30 40 50 60 70
sIL-6Rα/IL-6 Exposure Time (min)

Figure 3.3 Effect of A_{2A}AR gene transfer on STAT3 phosphorylation and expression in response to sIL6Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT3 and total STAT3 levels from three experiments is presented (***p<0.001 and *P<0.05 *versus* the response observed in AV.GFP-infected cells). Basal set at 100.


0 15 30 60 0 15 30 60
Incubation time (min)
+sIL-6Rα/IL-6
Mr
97
Phospho-STAT3
97
Total STAT3
AV.GFP
AV.mycA2AAR


Tyr705 STAT3 phosphorylation
(Fold stimulation over basal)
1200
1000
800
600
400
200
0
0 10 20 30 40 50 60 70
sIL-6Rα/IL-6 Exposure Time (min)
GFP PSTAT3
A2AAR PSTAT3
* * *
* * *
* * *

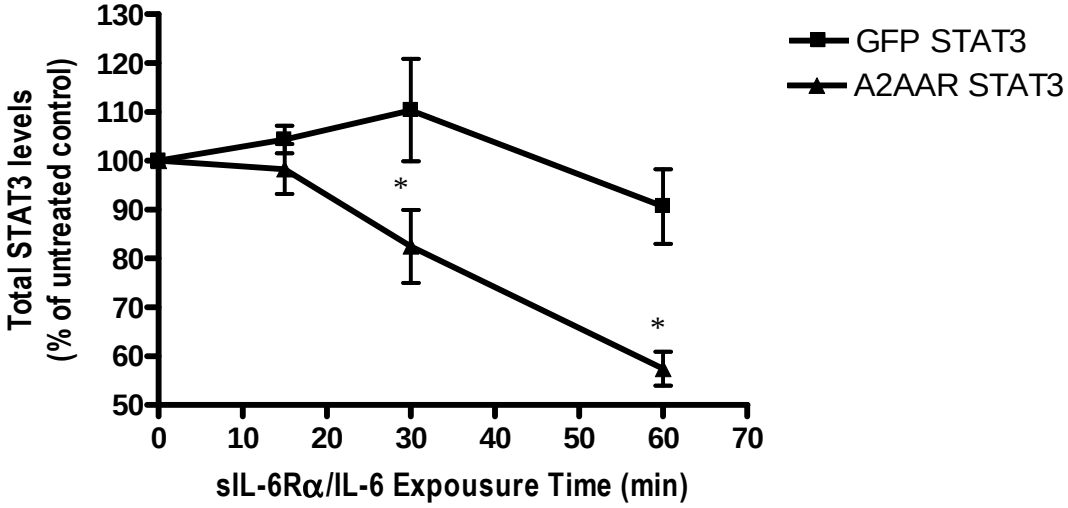
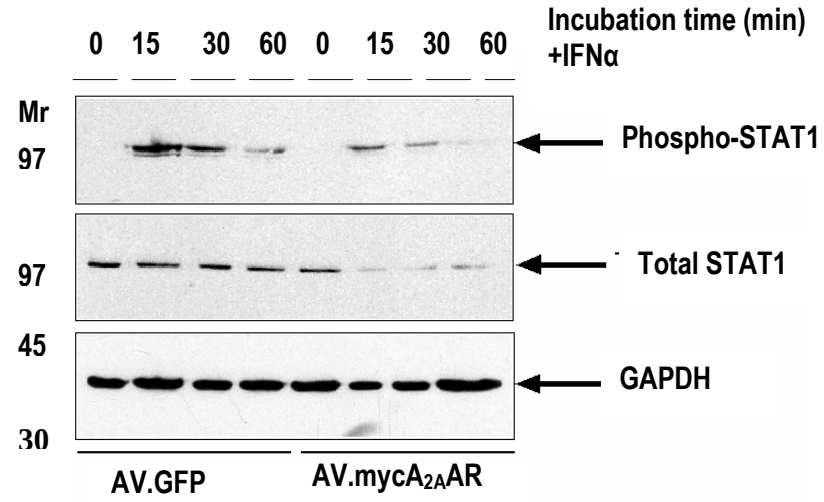
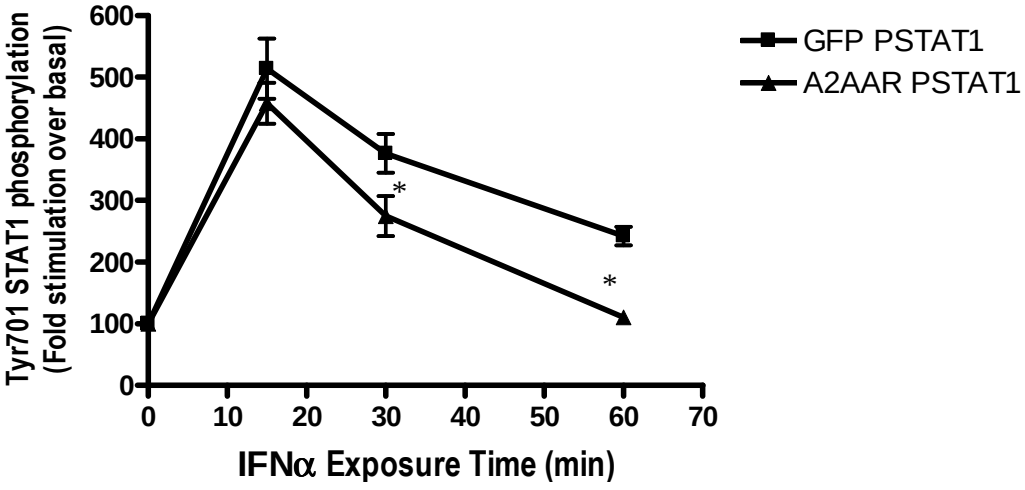

Total STAT3 levels
(% of untreated control)
130
120
110
100
90
80
70
60
50
0 10 20 30 40 50 60 70
sIL-6Rα/IL-6 Expousure Time (min)
GFP STAT3
A2AAR STAT3
*
*

Figure 3.4 Effect of A_{2A}AR gene transfer on STAT1 phosphorylation and expression in response to IFNα

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 500 unit/ml IFNα for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT1 and total STAT1 levels from three experiments is presented (***$p<0.001$ and *$p<0.05$ *versus* the response observed in AV.GFP-infected cells). Basal set at 100.


Incubation time (min)
+IFNα
0 15 30 60 0 15 30 60
Mr
97
Phospho-STAT1
97
Total STAT1
45
GAPDH
30
AV.GFP
AV.mycA2AAR


Tyr701 STAT1 phosphorylation
(Fold stimulation over basal)
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70
IFNα Exposure Time (min)
GFP PSTAT1
A2AAR PSTAT1
*
*

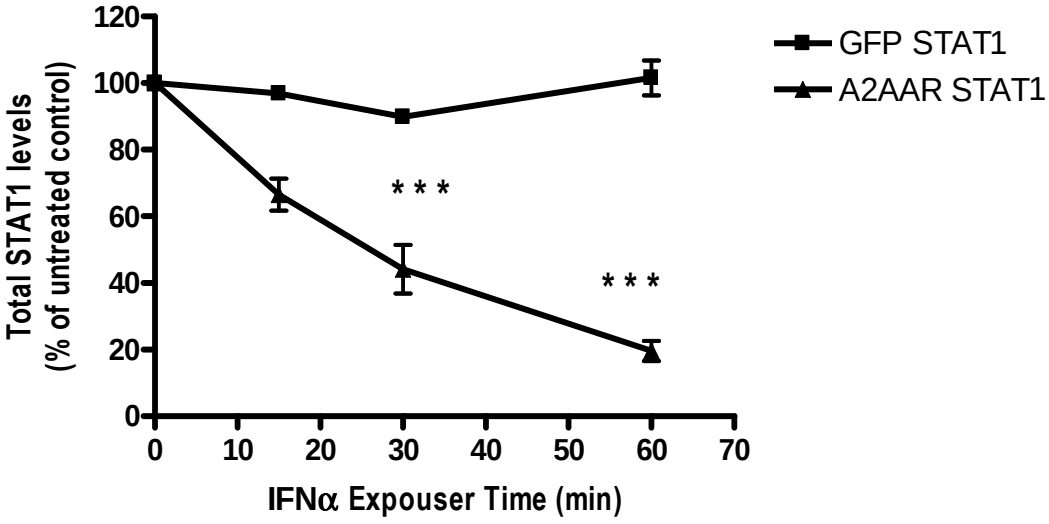
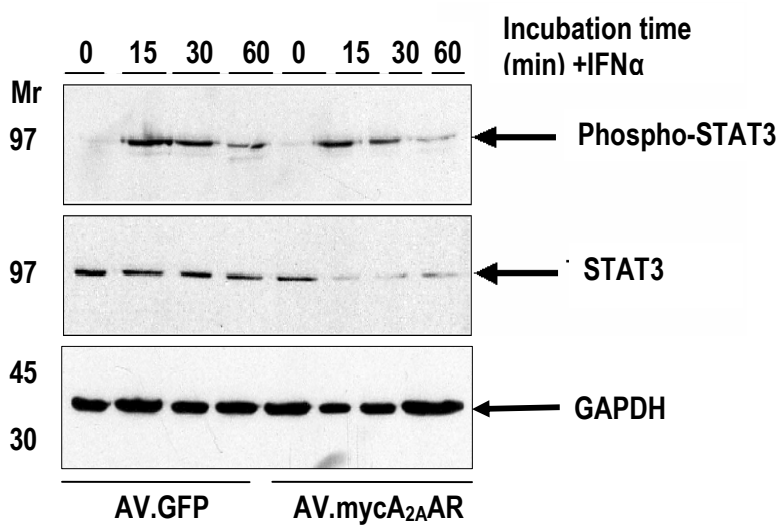
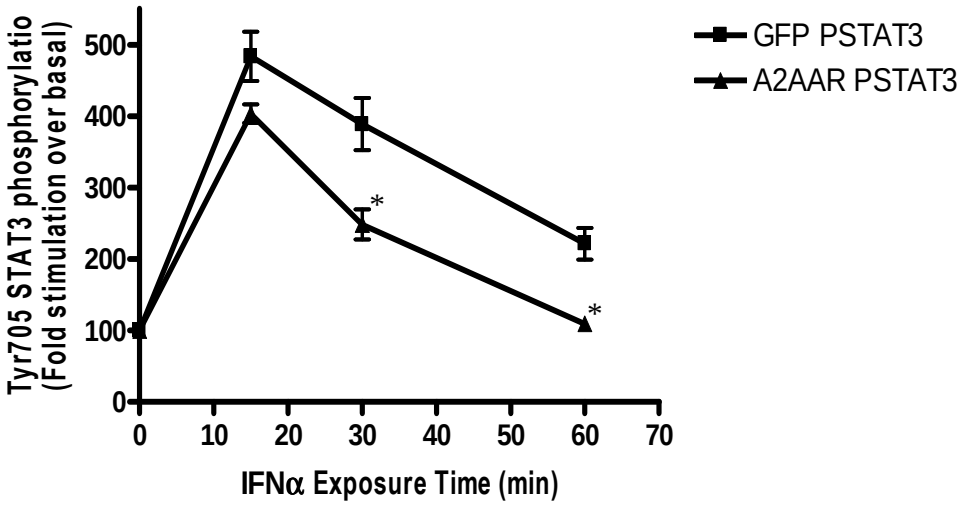

Total STAT1 levels
(% of untreated control)
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70
IFNα Expouser Time (min)
GFP STAT1
A2AAR STAT1
* * *
* * *

Figure 3.5 Effect of A_{2A}AR gene transfer on STAT3 phosphorylation and expression in response to IFNα

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 500 unit/ml IFNα for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT3 and total STAT3 levels from three experiments is presented (***$p<0.001$ and *$p<0.05$ *versus* the response observed in AV.GFP-infected cells). Basal set at 100.


Incubation time (min) +IFNα
0 15 30 60 0 15 30 60
Mr
97
Phospho-STAT3
97
STAT3
45
30
GAPDH
AV.GFP
AV.mycA2AAR


Tyr705 STAT3 phosphorylatio (Fold stimulation over basal)
500
400
300
200
100
0
0 10 20 30 40 50 60 70
IFNα Exposure Time (min)
GFP PSTAT3
A2AAR PSTAT3
*
*

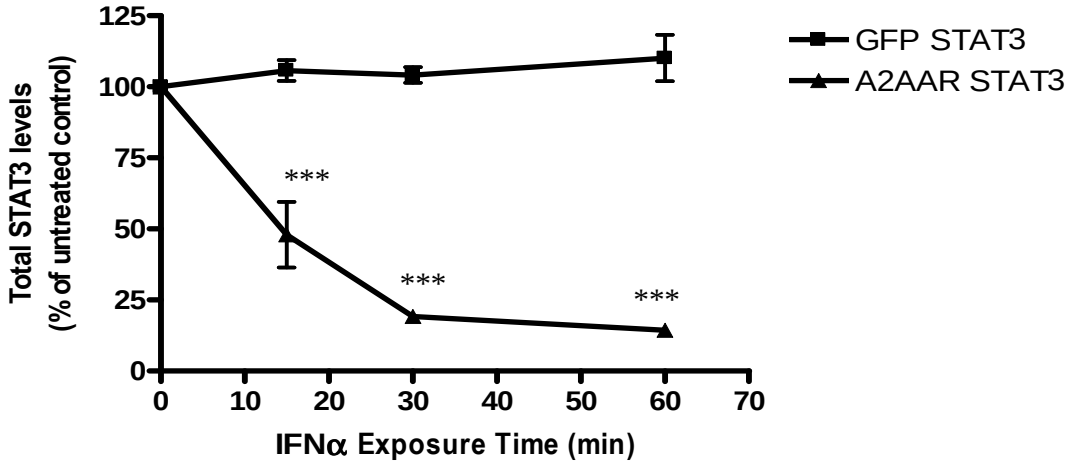
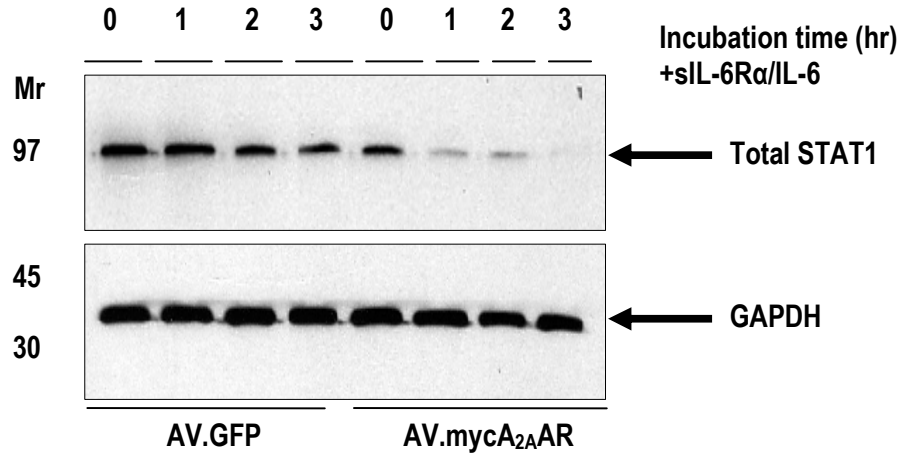

Total STAT3 levels (% of untreated control)
125
100
75
50
25
0
0 10 20 30 40 50 60 70
IFNα Exposure Time (min)
GFP STAT3
A2AAR STAT3
***
***
***

Figure 3.6 Effect of A_{2A}AR gene transfer on STAT1 expression in response to prolonged exposures to IL-6Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT1 levels from three experiments with each cytokine is presented (***p<0.001 *versus* the levels in AV.GFP-infected cells at the given time point). Basal set at 100.



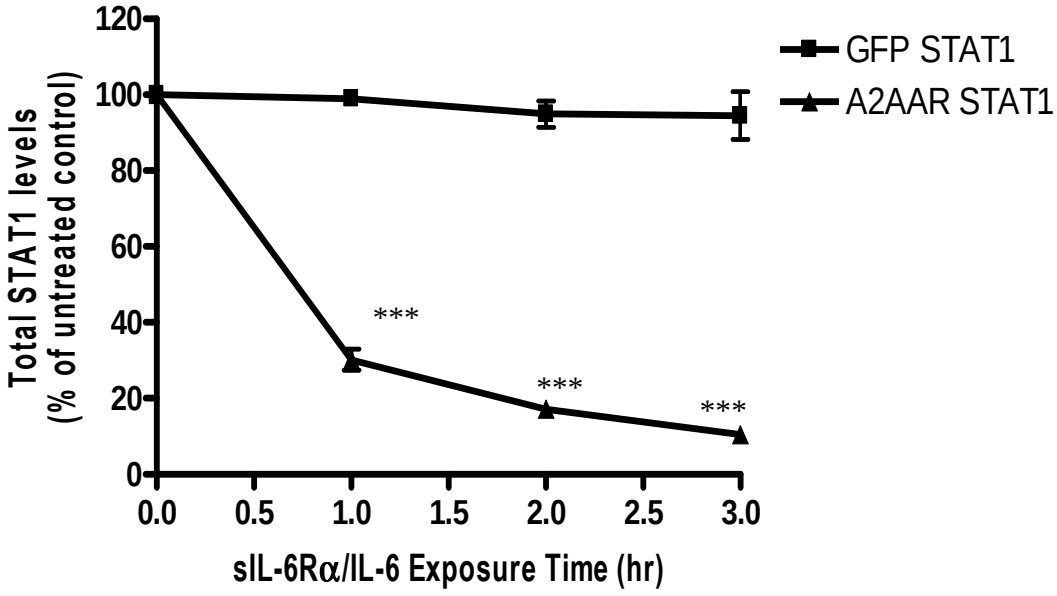
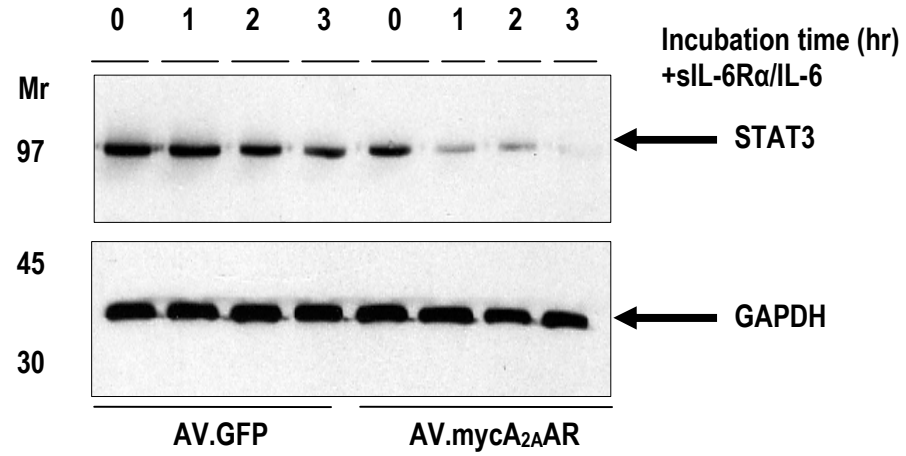


Figure 3.7 Effect of A_{2A}AR gene transfer on STAT3 expression in response to prolonged exposure to sIL-6Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels from three experiments with each cytokine is presented (***p<0.001 *versus* the levels in AV.GFP-infected cells at the given time point). Basal set at 100.



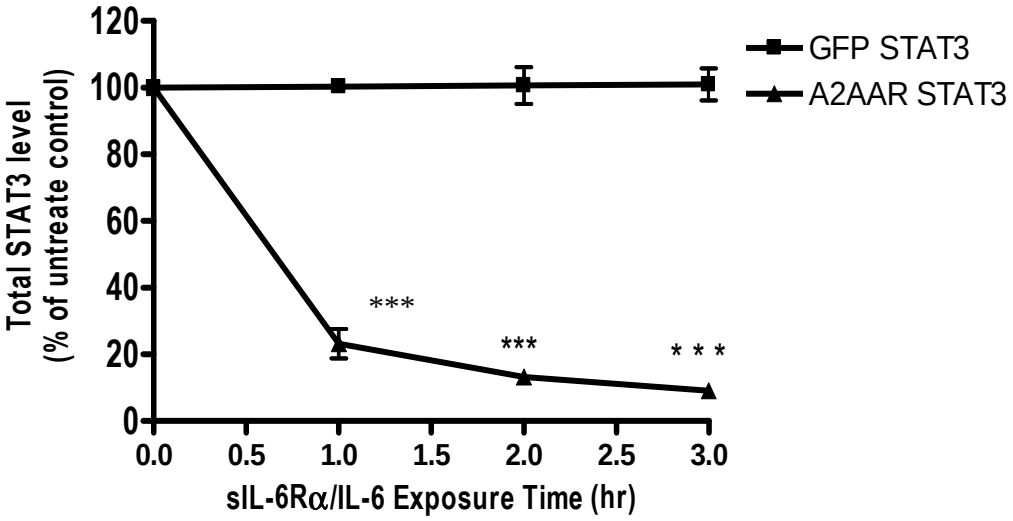
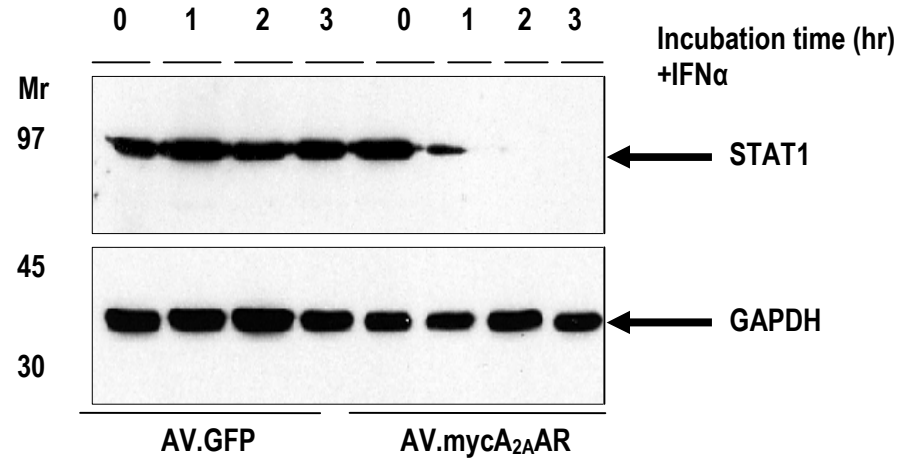


Figure 3.8 Effect of A_{2A}AR gene transfer on STAT1 expression in response to prolonged exposure to IFNα

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 500 unit/ml IFNα for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT1 levels from three experiments with each cytokine is presented (***p<0.001 *versus* the levels in AV.GFP-infected cells at the given time point). Basal set at 100.



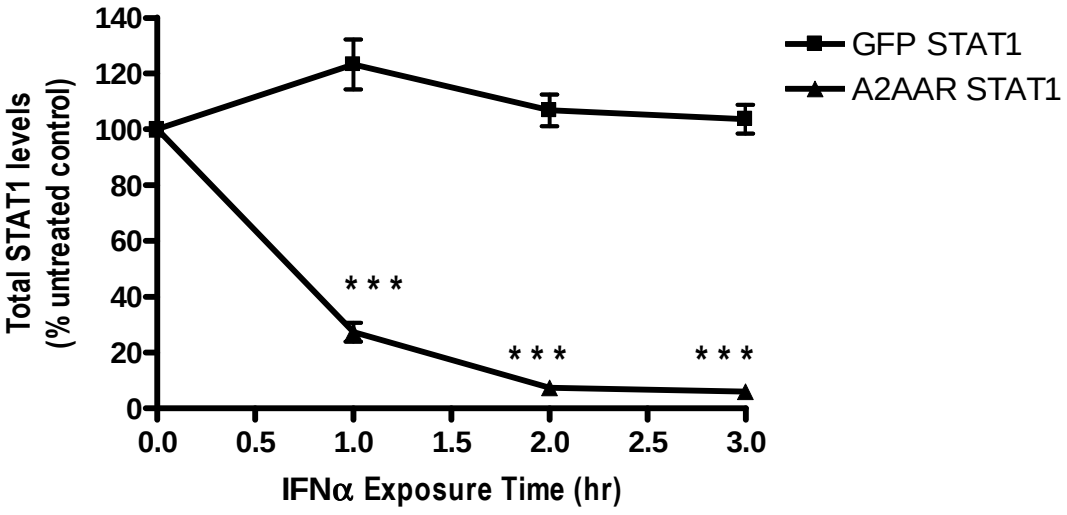
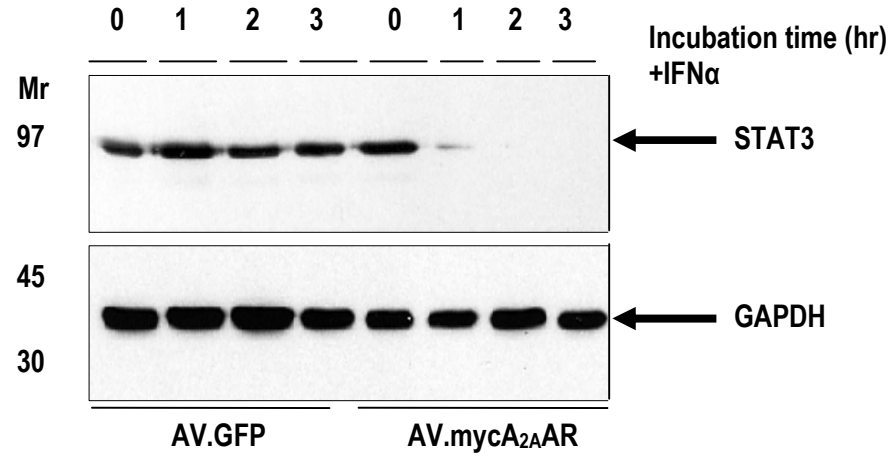


Figure 3.9 Effect of A_{2A}AR gene transfer on STAT3 expression in response to prolonged exposure to IFNα

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 500 unit/ml IFNα for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels from three experiments with each cytokine is presented (****p*<0.001 *versus* the levels in AV.GFP-infected cells at the given time point). Basal set at 100.



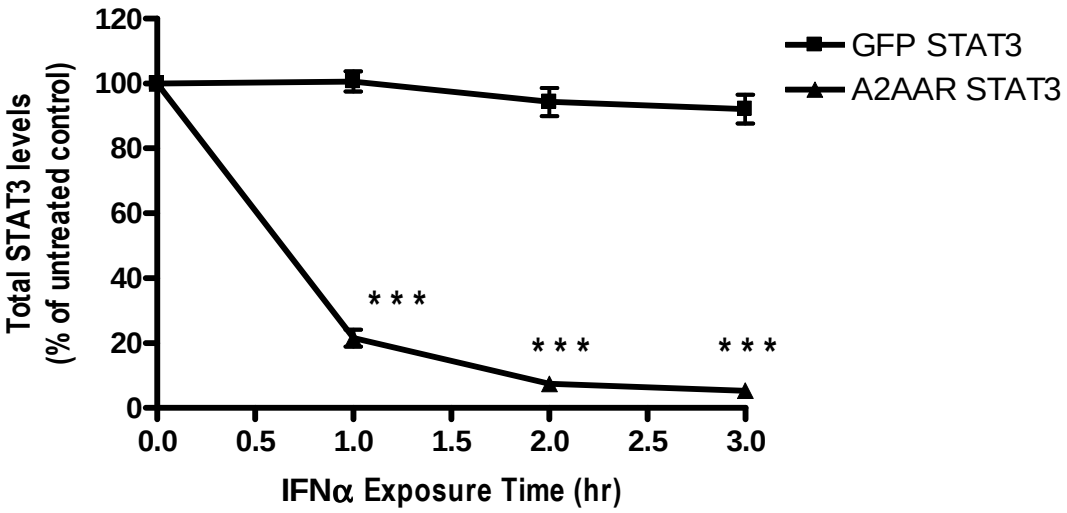
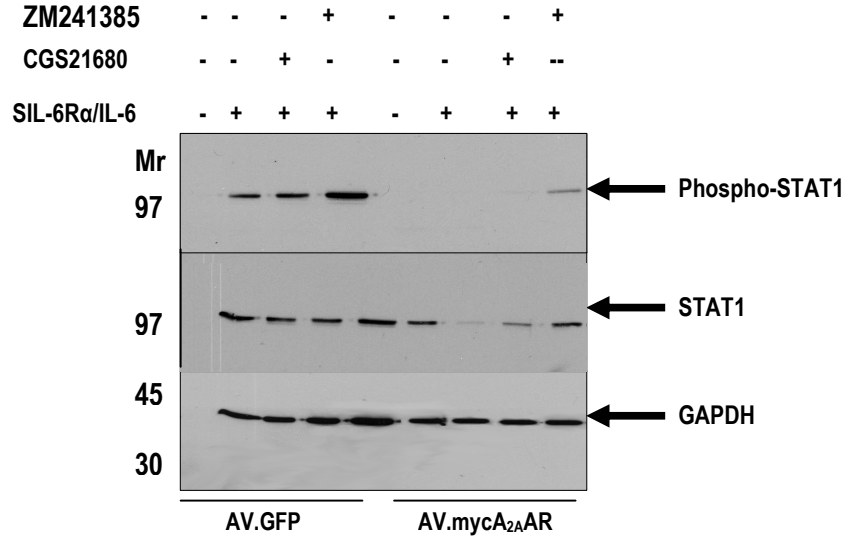
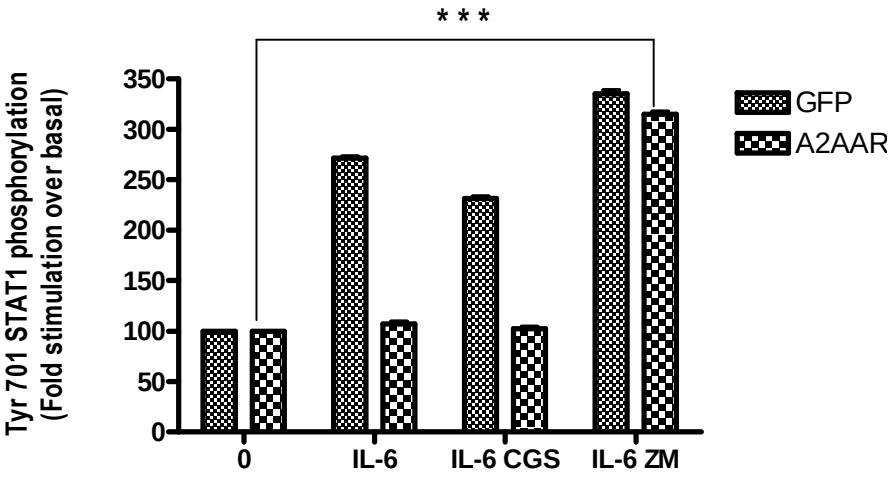


Figure 3.10 Effect of A_{2A}AR on STAT1 phosphorylation and expression in response to sIL-6 Rα/ IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding cells were infected with the indicated AVs prior to pre-treatment with or without 6μM CGS21680 or 1μM ZM241485 for 1hr and treatment with 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 1hr as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT1 and total STAT1 levels from four experiments is presented (***p<0.001 and **p<0.01 *versus* the levels in untreated AV.mycA_{2A}AR-infected cells). Basal set at 100.


ZM241385 - - - + - - - +
CGS21680 - - + - - - + --
SIL-6Rα/IL-6 - + + + - + + +
Mr
97
97
45
30
Phospho-STAT1
STAT1
GAPDH
AV.GFP
AV.mycA2AAR


* * *
Tyr 701 STAT1 phosphorylation
(Fold stimulation over basal)
350
300
250
200
150
100
50
0
0
IL-6
IL-6 CGS
IL-6 ZM
GFP
A2AAR

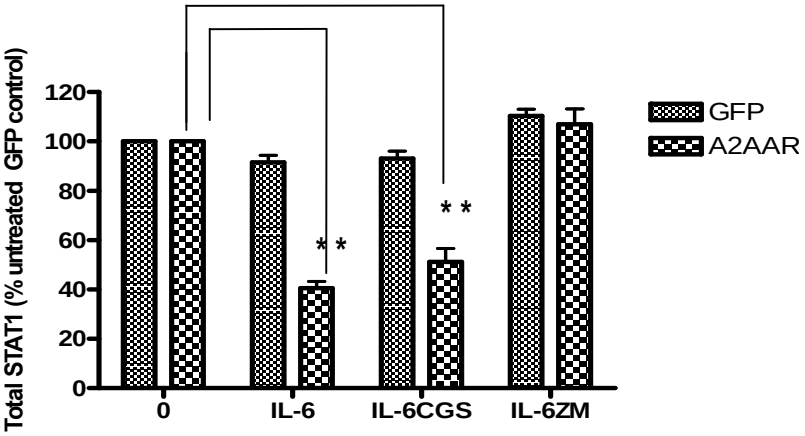
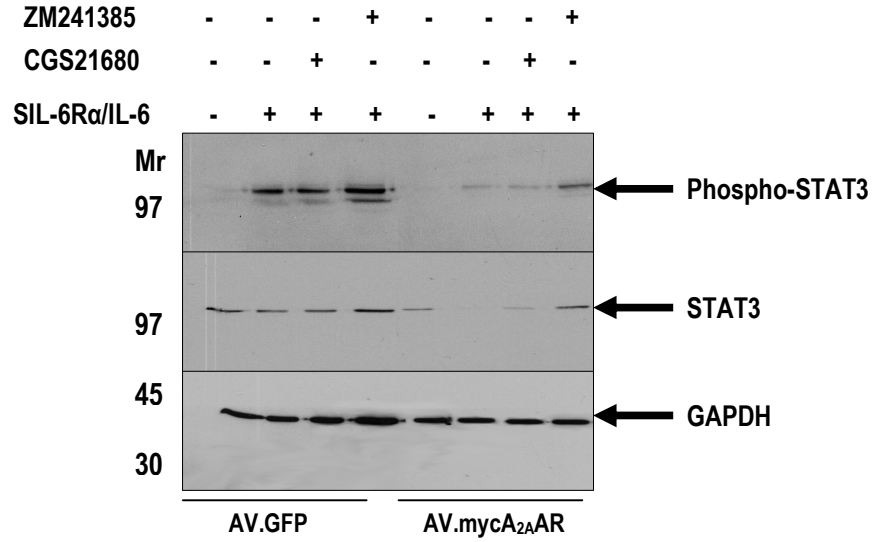

Total STAT1 (% untreated GFP control)
120
100
80
60
40
20
0
* *
* *
0
IL-6
IL-6CGS
IL-6ZM
GFP
A2AAR

Figure 3.11 Effect of A_{2A}AR on STAT3 phosphorylation and expression in response to sIL-6 Rα/ IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding cells were infected with the indicated AVs prior to pre-treatment with or without 6μM CGS21680 or 1μM ZM241485 for 1hr and treatment with 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 1hr as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT3 and total STAT3 levels from four experiments is presented (**p<0.01 *versus* the levels in untreated AV.mycA_{2A}AR-infected cells). Basal set at 100.


ZM241385 - - - + - - - +
CGS21680 - - + - - - + -
SIL-6Rα/IL-6 - + + + - + + +
Mr
97
97
45
30
Phospho-STAT3
STAT3
GAPDH
AV.GFP
AV.mycA2AAR

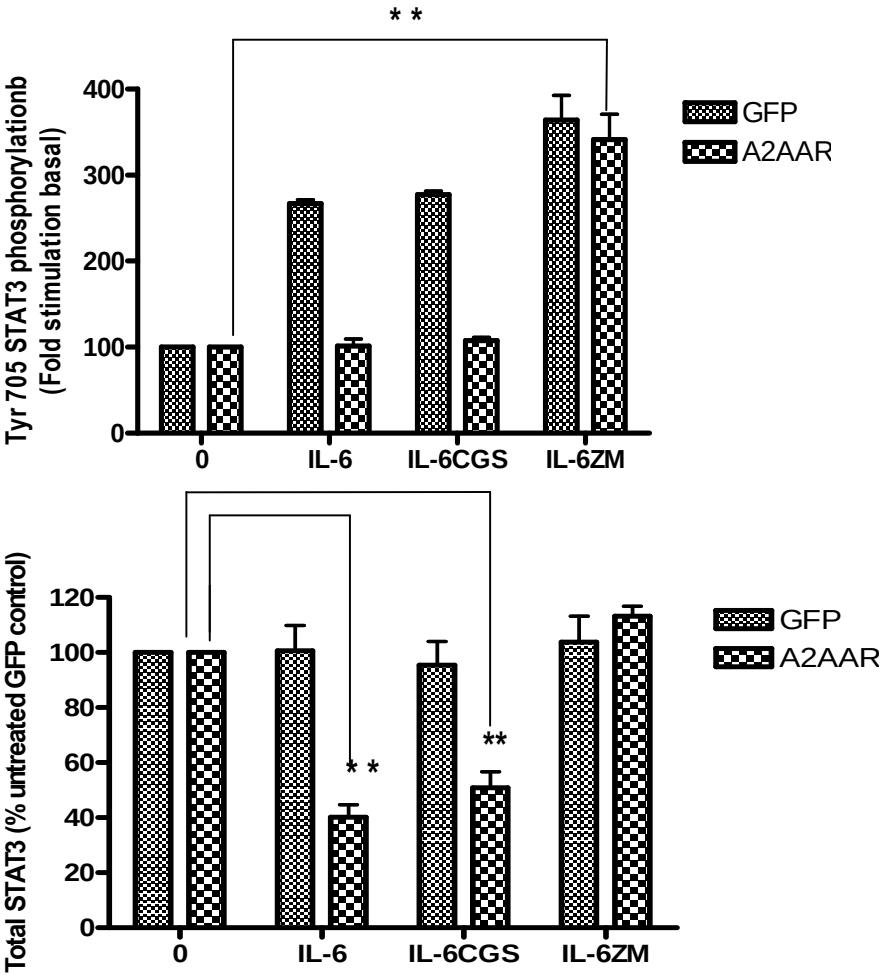

* *
Tyr 705 STAT3 phosphorylationb (Fold stimulation basal)
400
300
200
100
0
GFP
A2AAR
0
IL-6
IL-6CGS
IL-6ZM
Total STAT3 (% untreated GFP control)
120
100
80
60
40
20
0
* *
**
GFP
A2AAR
0
IL-6
IL-6CGS
IL-6ZM

Figure 3.12 Effect of A_{2A}AR expression and sIL-6Rα/IL-6 expression on HUVECs viability

HUVECs were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 3 hrs or 0.1% (w/v) sodium azide for 16 hrs. Cell viability was then determined by measurement of MTT reduction. This is one of three experiments that produced similar data.

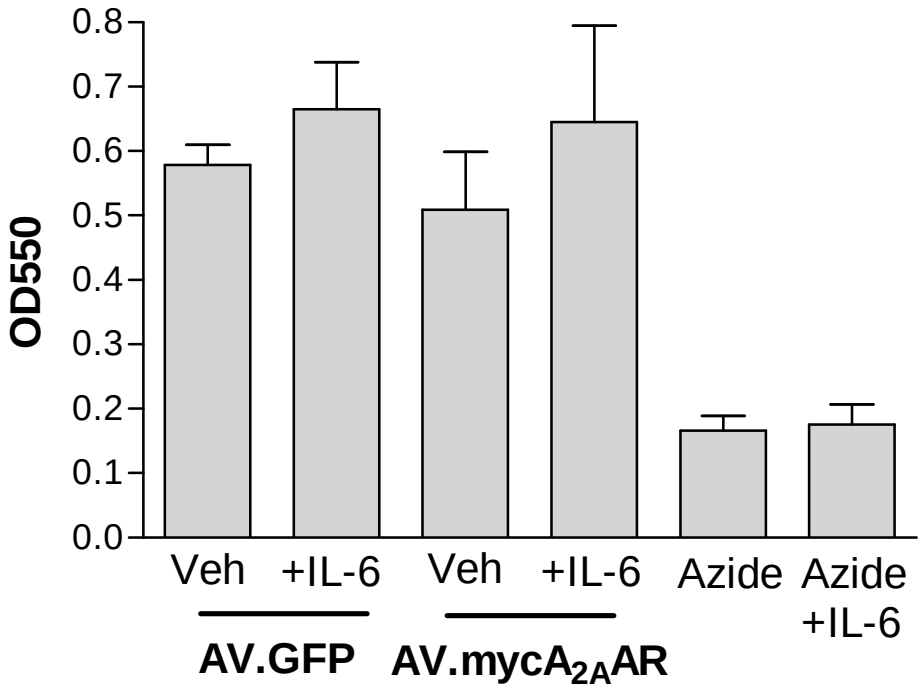


Figure 3.13 Effect of MG132 on sIL-6Rα/IL-6-induced STAT1 downregulation in A_{2A}AR-expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 25ng/ml sIL-6 Rα/5ng/ml IL-6 up to 3hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT1 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT1 levels in vehicle-pretreated cells at the given time point). Basal set at 100.

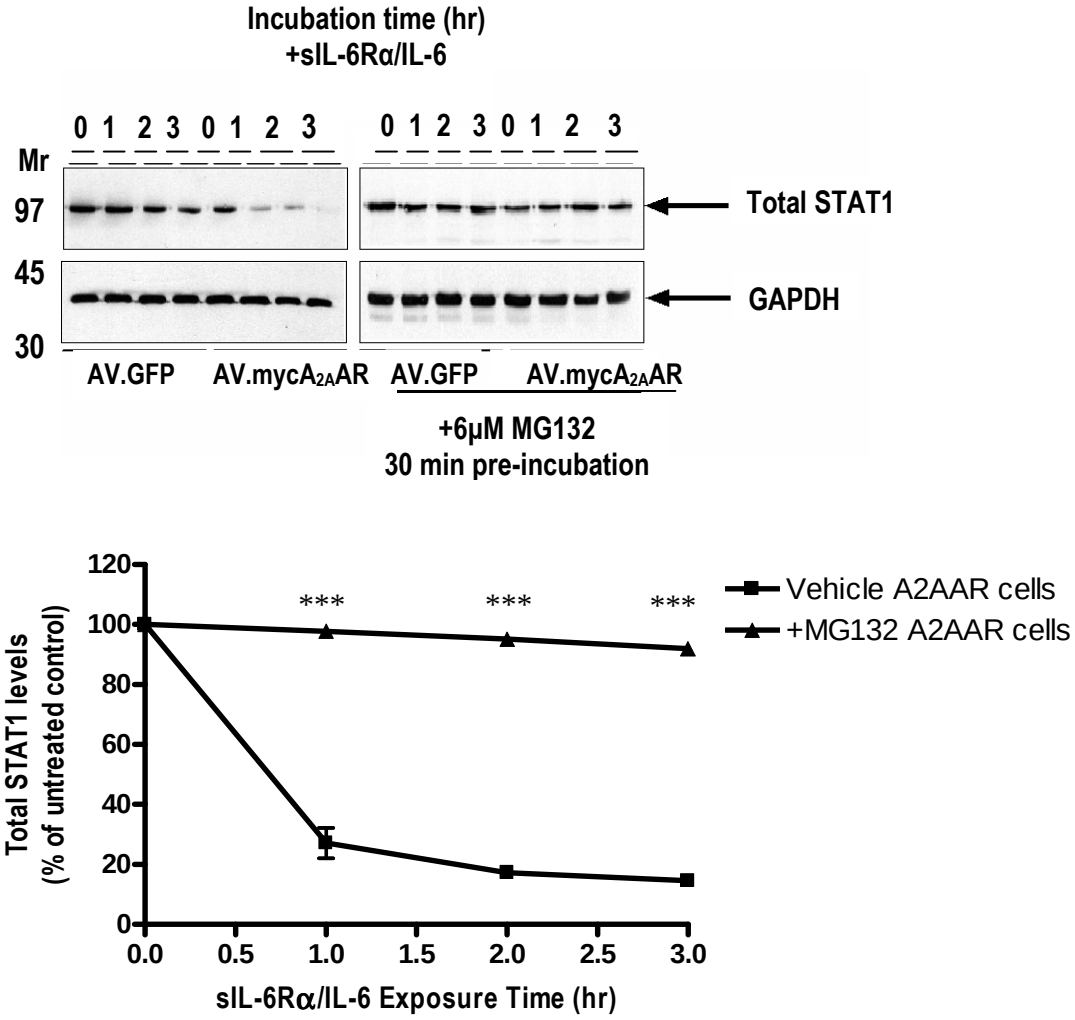


Figure 3.14 Effect of MG132 on sIL-6Rα/IL-6-induced STAT3 downregulation in A_{2A}AR-expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 25ng/ml sIL-6 Rα/5ng/ml IL-6 up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT3 levels in vehicle-pretreated cells at the given time point). Basal set at 100.

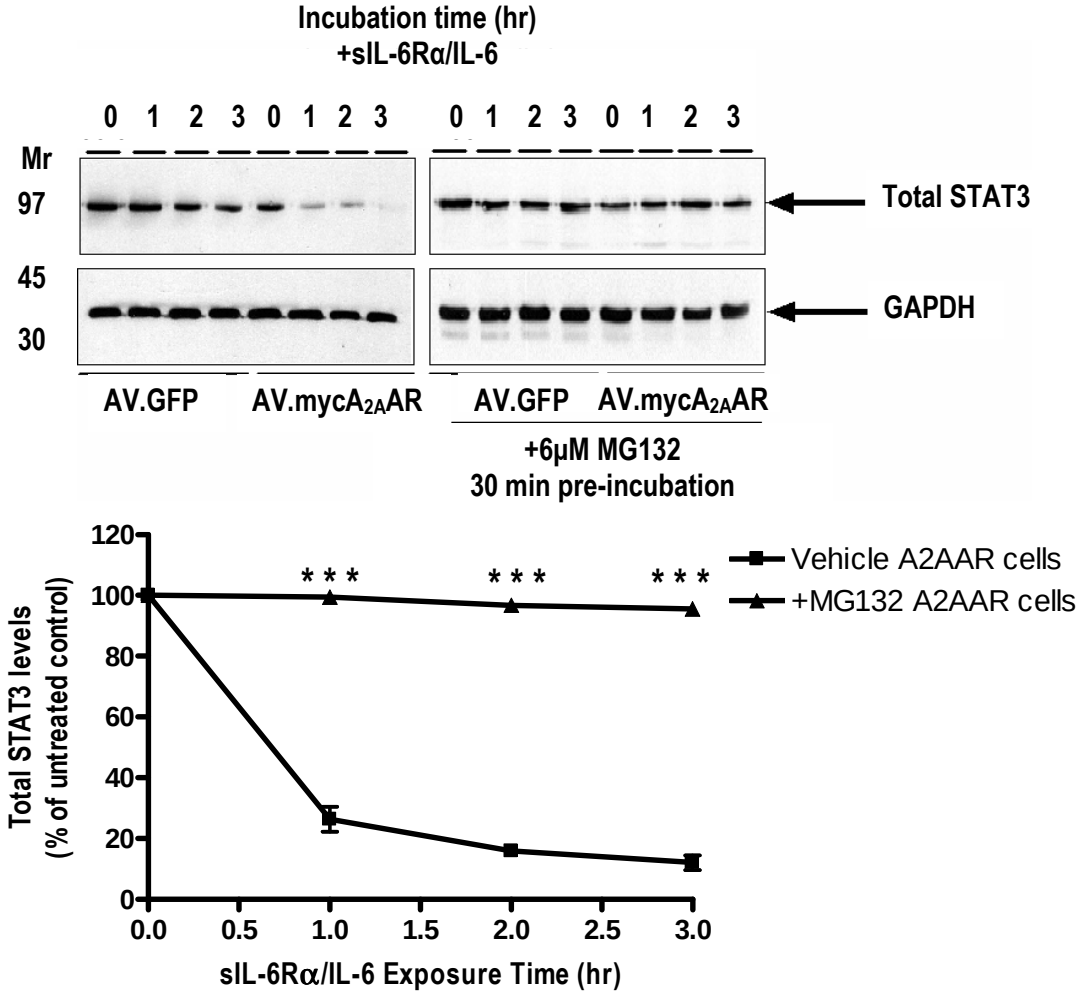
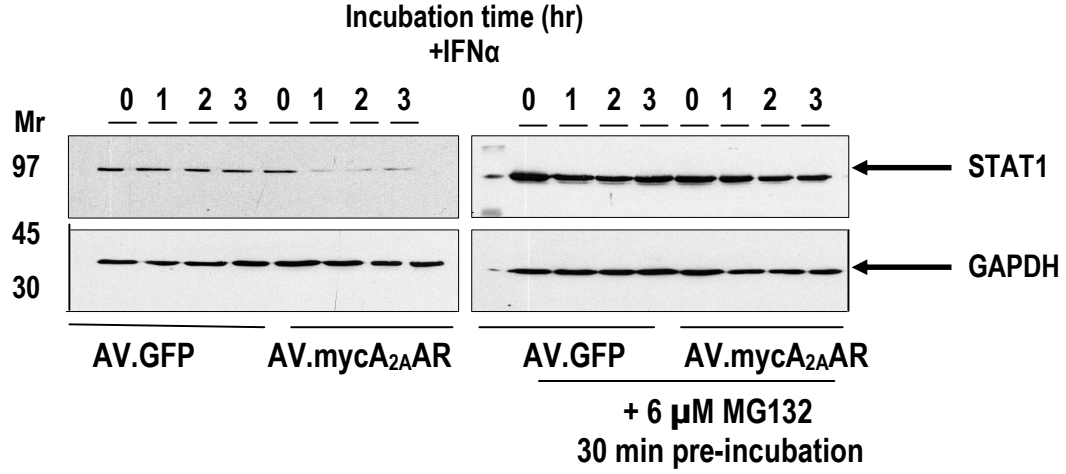


Figure 3.15 Effect of MG132 on IFNα-induced STAT1 downregulation in A_{2A}AR-expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 500 unit/ml IFNα up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT1 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT1 levels in vehicle-pretreated cells at the given time point). Basal set at 100.



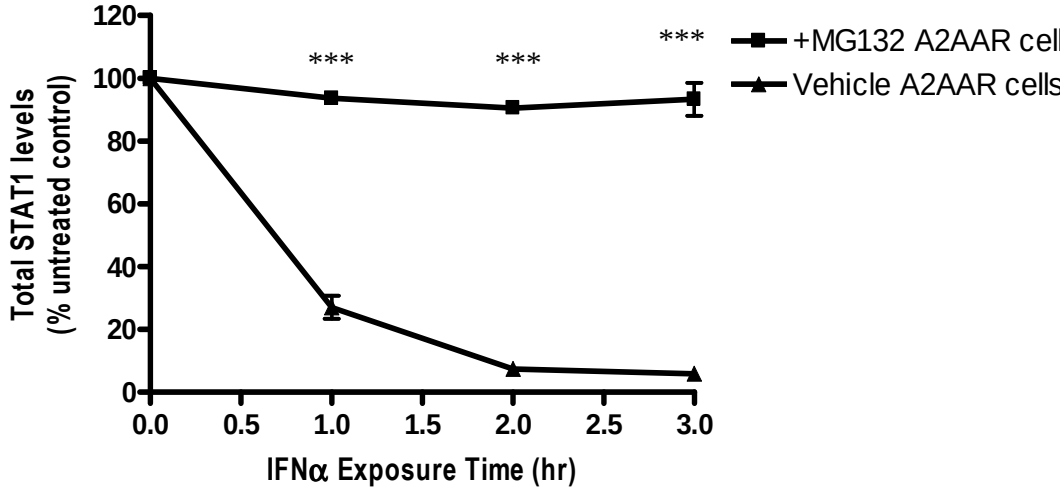


Figure 3.16 Effect of MG132 on IFNα-induced STAT3 downregulation in A_{2A}AR-expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 500 unit/ml IFNα up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT3 levels in vehicle-pretreated cells at the given time point). Basal set at 100.

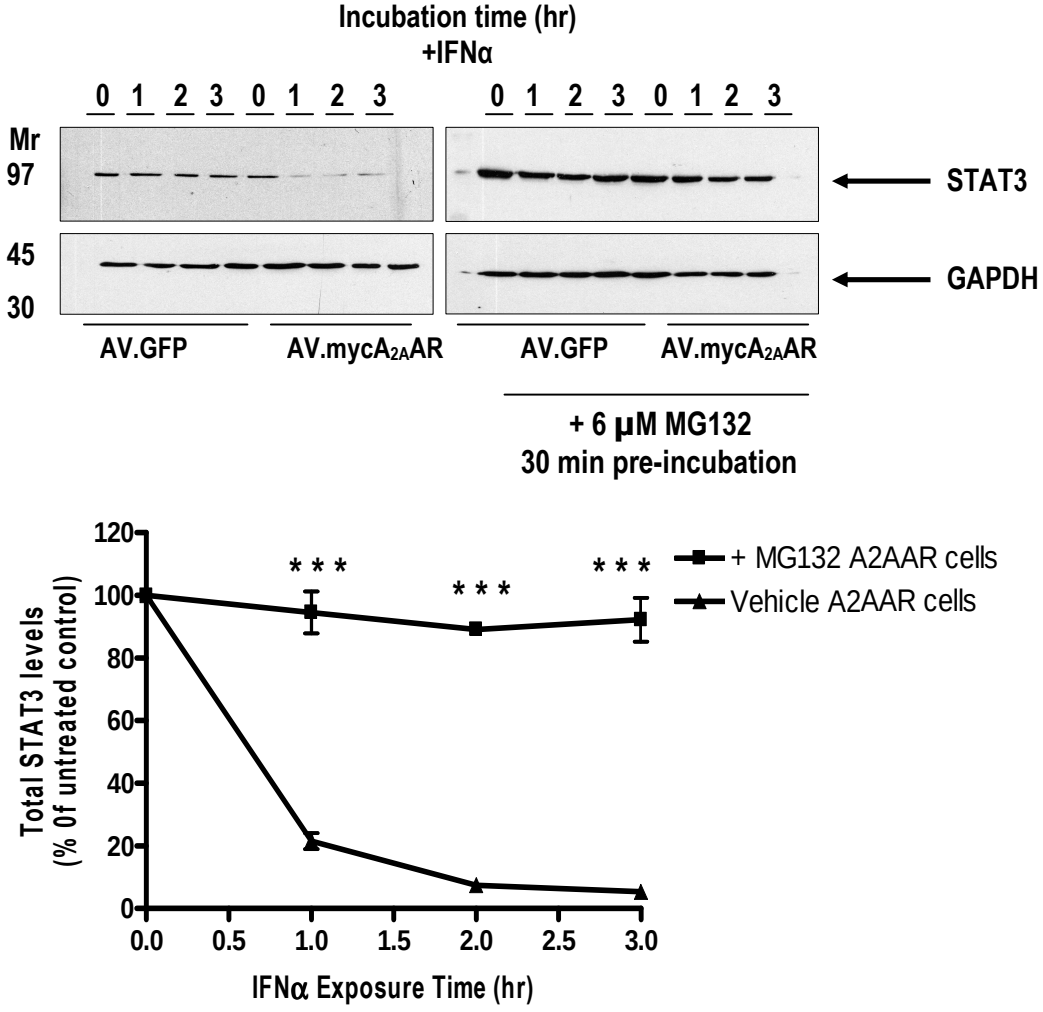
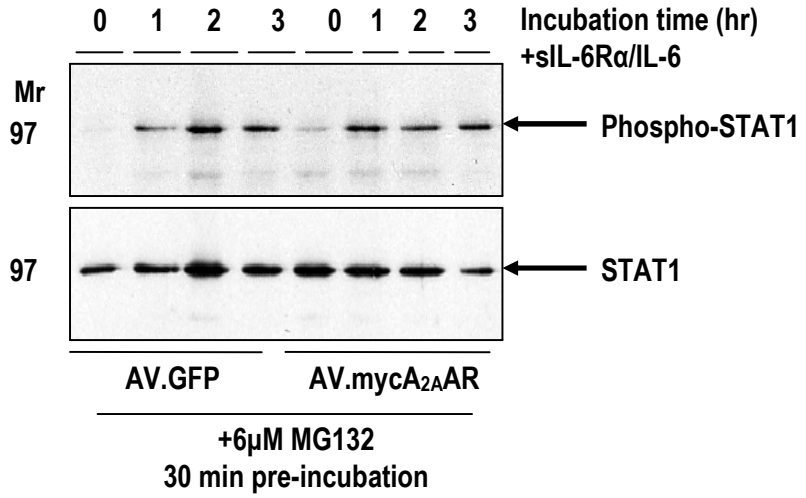


Figure 3.17 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT1 phosphorylation in response to sIL-6 Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 25ng/ml sIL-6 Rα/5ng/ml IL-6 up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT1 levels from three experiments is presented. Basal set at 100.



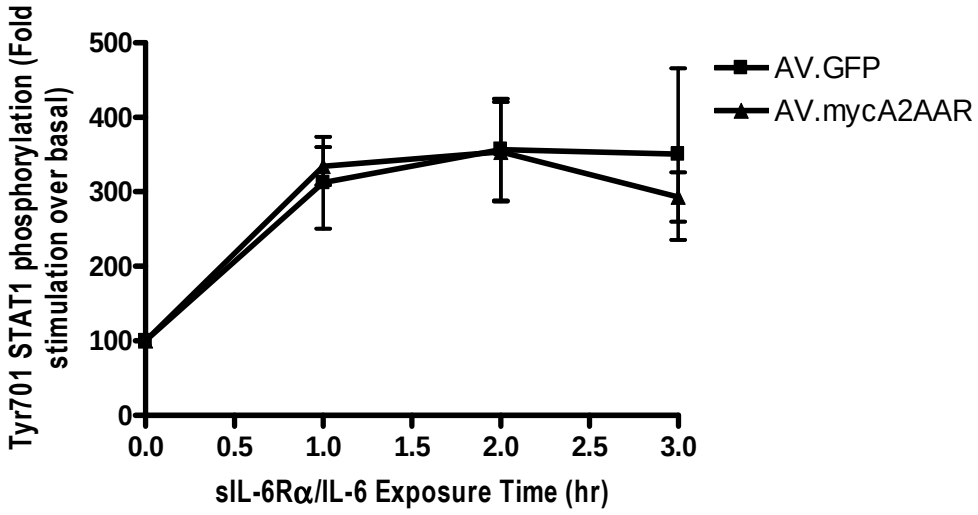
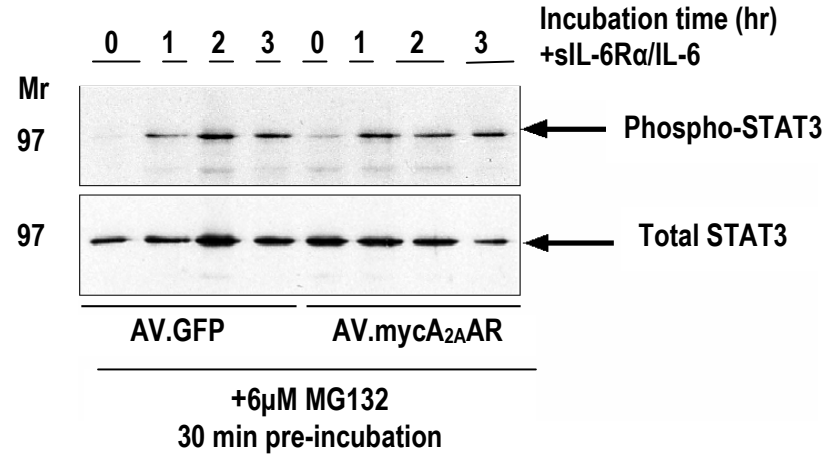


Figure 3.18 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT3 phosphorylation in response to sIL-6 Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 25ng/ml sIL-6 Rα/5ng/ml IL-6 up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT3 levels from three experiments is presented. Basal set at 100.



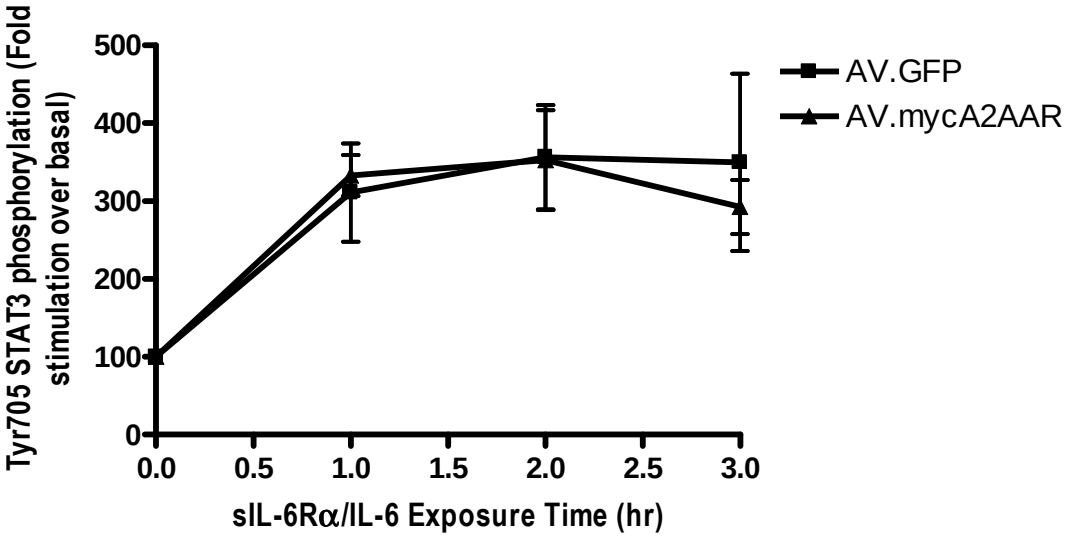


Figure 3.19 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT1 phosphorylation in response to IFNα

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 500 unit/ml IFNα up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT1 levels from three experiments is presented. Basal set at 100.

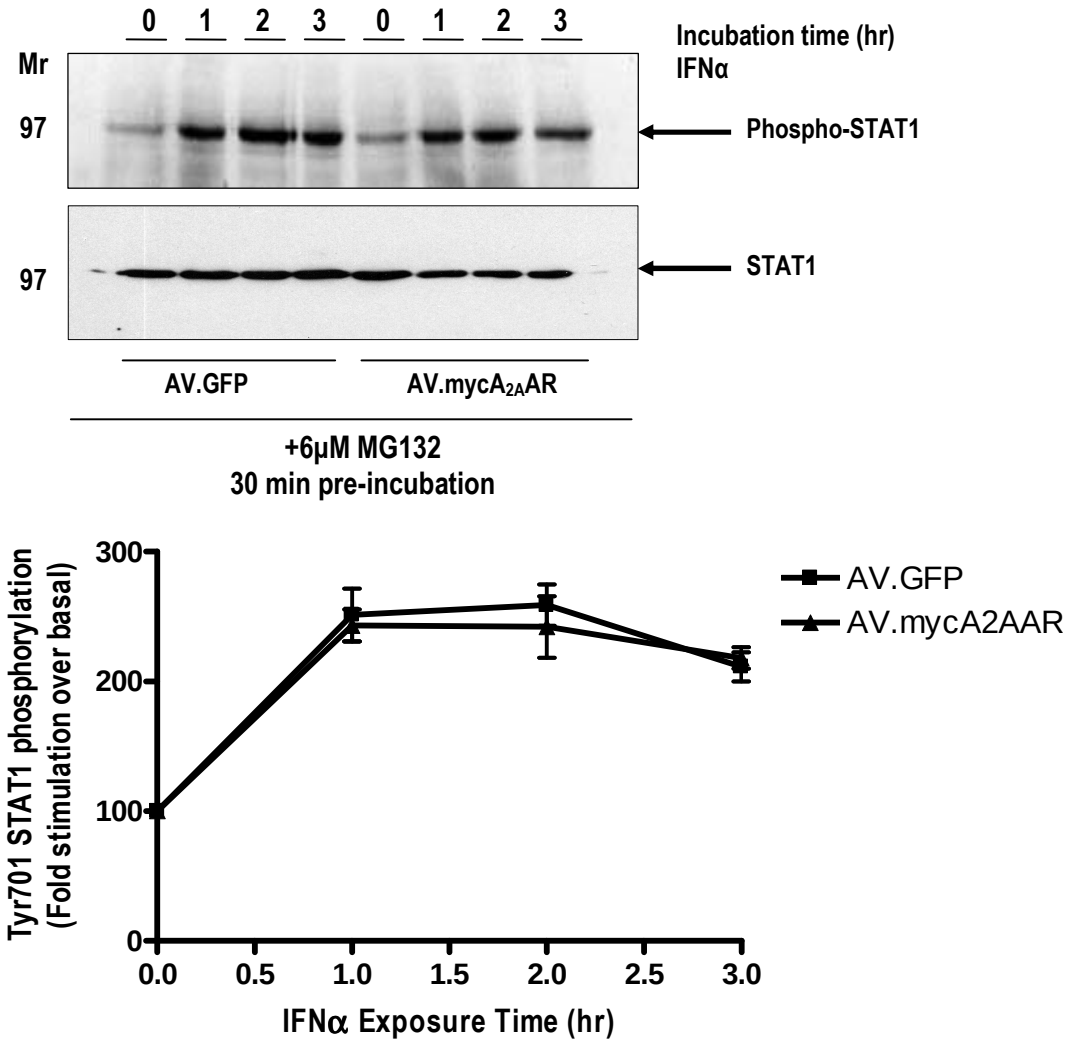
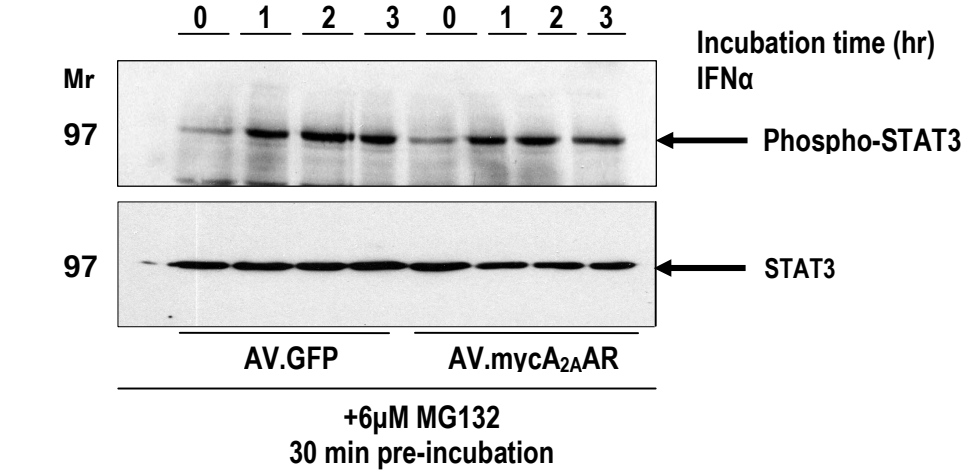
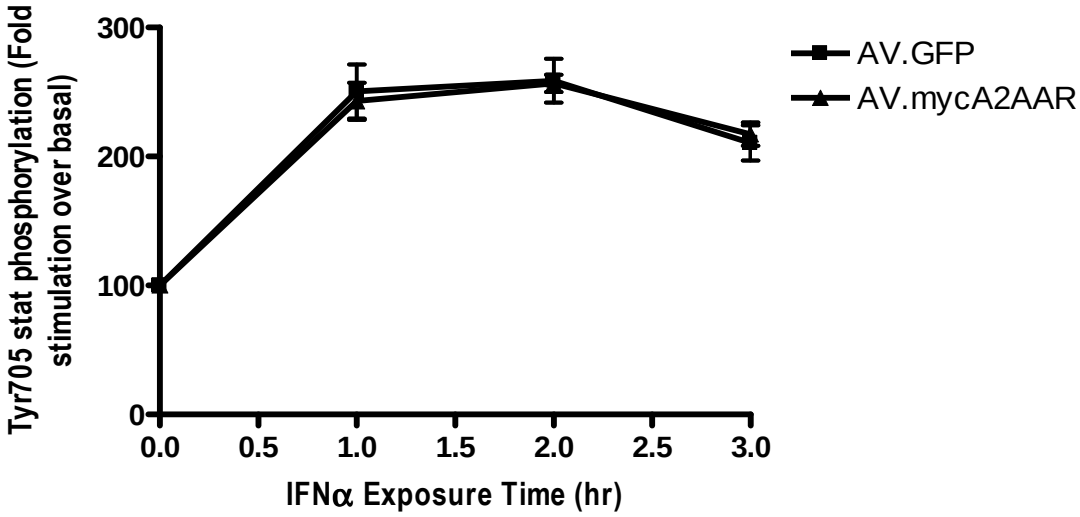


Figure 3.20 Blocking degradation abolishes the inhibitory effect of the A_{2A}AR on STAT3 phosphorylation in response to IFNα

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and treatment with 500 unit/ml IFNα up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT3 levels from three experiments is presented. Basal set at 100.





3.3 Discussion

Numerous pharmacological studies have identified the A_{2A}AR as a protective anti-inflammatory G-protein-coupled receptor (Lappas *et al.*; 2005). Gene dosage studies have provided evidence to show that, at least in T-lymphocytes, there is no A_{2A}AR reserve (Armstrong *et al.*; 2001). Consequently, pathophysiological conditions that alter A_{2A}AR expression, such as the onset of hypoxia (Kobayashi and Millhorn; 1999) and EC exposure to Th1 cytokines (Nguyen *et al.*; 2003), are likely to alter cellular responsiveness to inflammatory stimuli. However, despite unequivocal evidence of its potent anti-inflammatory properties across different cell types, only the ability of the receptor to suppress NF-κB activation has been offered as an explanation for its effects to date (Majumdar and Aggarwal; 2003; Sands *et al.*; 2004).

This study has extended these observations by demonstrating that the A_{2A}AR overexperession can prime cytokine-activated STATs for degradation by the proteasome. Similar to the effect observed on suppression of NF-κB in two separate cell systems (Sands *et al.*; 2004), overexpression of the A_{2A}AR in the absence of any added agonist was sufficient to prime STATs for degradation (Figure 3.2-3.9). Thus, the receptor may be sufficiently active in the absence of agonist to trigger its associated signalling pathways. This phenomenon has been observed for both endogenous receptors and overexpression studies of GPCRs (Bond and Ijzerman; 2006; Vischer *et al.*; 2006). Reversal of the effect of receptor over-expression on STAT down-regulation by the A_{2A}AR-selective antagonist ZM241385 (Figure 3.10 and 3.11) is consistent with this hypothesis. Therefore, "constitutive" activation of the A_{2A}AR, whether due to high agonist-independent activity or elevation in locally generated adenosine levels, may represent an especially important aspect of its function. Moreover, the effect is not restricted to overexpressed A_{2A}ARs, since exposure of low levels of endogenous A_{2A}AR in HUVECs with ZM241385 also potentiates sIL-6Rα/IL-6-stimulated phosphorylation of STAT3 (W.Sands

unpublished data). Endothelial cells are an abundant source of adenosine *in vitro*, partly due to expression of CD39 which converts adenosine triphosphate/adenosine diphosphate (ATP/ADP) to adenosine monophosphate (AMP) and CD73 which converts AMP to adenosine (Eltzschig *et al.*; 2004; Zernecke *et al.*; 2006). Both CD39 and CD73 are each strongly induced upon the onset of hypoxia, and CD73 expression can also be potentiated by IFN-α and adenosine itself via receptor-mediated elevation of cAMP (Narravula *et al.*; 2000; Niemela *et al.*; 2004). Thus, control of adenosine accumulation and initiation of protective signalling clearly represents an important adaptive mechanism by which hypoxia-mediated damage to the endothelium is minimised. The resulting accumulation of adenosine in large blood vessels is thought to play an important protective role *in vivo* by limiting endothelial cell activation and subsequent monocyte attachment (Zernecke *et al.*; 2006).

To begin identifying potential mechanisms by which A_{2A}AR overexpression could impact on cytokine signalling, we have been examining the effects of A_{2A}AR on sIL-6Rα/IL-6 and IFNα signalling in vascular ECs and assessing its functional significance. A_{2A}AR overexpression led to the more rapid degradation of STAT1 and STAT3 upon sIL-6Rα/IL-6 and IFNα stimulation. To determine whether STATs are targeted for degradation by the proteasome, experiments were performed to investigate the effect of proteasome inhibitor MG132 on the ability of A_{2A}AR overexpression to prime STATs for cytokine-mediated down-regulation. Figure 3.13-3.16 show that MG132 was sufficient to abolish the effect of A_{2A}AR overexpression on priming STATs for down-regulation in response to cytokines. Moreover, results in this Chapter show that blocking of proteasomal degradation with MG132 abolished A_{2A}AR inhibition of both sIL-6Rα/IL-6 and IFNα-stimulated phosphorylation of STATs. Cross-talk between cAMP-mediated signalling pathways and STAT activation has been previously reported by Sengupta *et al.* (1996), and suggests that A_{2A}AR-mediated inhibition of STAT is cAMP-dependent. In addition, studies by Ivashkiv *et al.* (1996) have shown that in T cell activation, cAMP can inhibit accumulation of STAT1 protein. The A_{2A}AR is highly expressed within most cells of

the immune system, platelets, heart, lung and endothelium (Fredholm *et al.*; 2001) . Classically, the A_{2A}AR signals via the G_s family of G proteins leading to an activation of adenylyl cyclase and the generation of cAMP. It is thought to mediate the vast majority of its intracellular effects by binding and activating cAMP-dependent protein kinase (PKA), which controls the phosphorylation status and activity of multiple intracellular substrates (Tasken and Aandahl; 2004). However, another family of intracellular cAMP sensors termed Epacs have also been identified (de Rooij *et al.*; 1998). Epac1 and -2 function as cAMP-activated guanine nucleotide exchange factors specific for the Rap family of small G proteins and, thus promote the accumulation of active GTP-bound Rap1 and -2 (Rooij *et al.*; 1998; Bos; 2003). Interestingly, a role for Epac in EC function was recently revealed by the finding that the Epac-mediated activation of Rap1 and the subsequent formation of adherens junctions contribute toward the ability of cAMP to enhance endothelial barrier function, an important aspect of its anti-inflammatory effects in vascular ECs (Cullere *et al.*; 2005; Fukuhara *et al.*; 2005). Previous work undertaken in our lab has shown that inhibition of STAT phosphorylation and induction of SOCS3 can occur via a cAMP/Epac/Rap1 pathway to limit IL-6 receptor signalling (Sands *et al.*; 2006). In addition, new evidence indicates that increased cAMP levels inhibit angiotensin II-mediated JAK/STAT3 activation by a variety of mechanisms. Interestingly, cAMP-elevating agents were shown to inhibit IL-6-induced STAT activation in monocytes (Sengupta *et al.*; 1996). Together, these data establish a link for crosstalk between cAMP and JAK/STAT3, although at present, it is not known how cAMP inhibits STAT activation. In addition, in this study we showed that inhibition of proteasome function was sufficient to block the effect of A_{2A}AR overexpression on STAT degradation. This demonstrates that priming of STAT1 and STAT3 for degradation is the only mechanism responsible for the down regulation of STATs upon cytokine stimulation in our model.

In summary, many different mechanisms have been implicated in downregulating STAT1 and STAT3 signalling. However, for many of these the molecular mechanisms have not been well characterised. Several cytoplasmic tyrosine phosphatases, including tyrosine phosphatases containing a SH2 domain (SHP-1), CD45, and protein tyrosine phosphatase 1B, are implicated in the dephosphorylation of STAT1 and STAT3. In addition, SOCS are potentially key negative regulatory modulators of STAT1 and STAT3 signalling as they bind to Tyr phosphorylated cytokine receptors and JAK catalytic sites. Moreover, the protein PIAS3 was found to be specific for the inhibition of activated STAT3 (Chung *et al.*; 1997a). Scaffolding proteins such as the STAT3-interacting protein (StIP1), a novel protein consisting of 12 WD40 repeats, can also inhibit STAT3 signalling by modulating the formation of multiprotein complexes that are central in the regulation of signal transduction, transcription, and targeted proteolysis. (Collum *et al.*; 2000) demonstrated that StIP1 had a high affinity for unphosphorylated JAK and STAT3, and when overexpressed blocks STAT3 activation and dimerisation/DNA binding, nuclear translocation, and reporter gene transcription following stimulation with IL-6.

Data in this Chapter reveals several important aspects of A_{2A}AR overexpression on cytokine and JAK-STAT signalling pathway. These include:

(1) Potentiation of A_{2A}AR function by increasing its overexpression reduces cytokine receptor activation of the JAK-STAT pathway by priming Tyr-phosphorylated STATs for proteasomal degradation.

(2) Over-expression of the A_{2A}AR in HUVECs suppresses the ability of IFNα and a sIL-6Rα/IL-6 trans-signalling complex to promote Tyr-phosphorylation of STATs 1 and 3 by targeting cytokine-activated STATs for proteasomal degradation.

(3) Pre-incubation with the proteasome inhibitor MG132 was sufficient to abolish the effect of A_{2A}AR overexpression on priming both STAT1 and STAT3 for down-regulation in response to sIL-6Rα/IL-6 and IFNα.

(4) The results show that inhibition of proteasome function was sufficient to block the inhibitory effect of the A_{2A}AR overexpression on STAT3 phosphorylation, demonstrating that priming of STATs for degradation is the only mechanism responsible for the reduced cytokine-stimulated STAT phosphorylation observed in A_{2A}AR-overexpressing cells.

(5) The effect of A_{2A}AR overexpression on STAT1 and STAT3 phosphorylation and down regulation could be rescued by co-incubation of sIL-6Rα/IL-6 with the A_{2A}AR-selective antagonist ZM241385. Conversely, co-incubation with the selective agonist CGS21680 did not further potentiate the effect of receptor expression to down regulate STAT in A_{2A}AR-overexpressing cells.

(6) GFP- and A_{2A}AR-overexpressing HUVECs had similar viabilities that were not affected by sIL-6Rα/IL-6 exposure at a 3 hr time point at which STAT degradation was detectable only in A_{2A}AR-overexpressing cells.

The results in this chapter have demonstrated that adenovirus-mediated human A_{2A}AR gene transfer to vascular ECs *in vitro* was sufficient to prime Tyr-phosphorylated STATs for proteasomal degradation upon stimulation of sIL-6Rα/IL-6 and IFNα. Both this study and others describing agonist-independent signalling from the A_{2A}AR have been cell systems in which the recombinant receptor is overexpressed (Chen *et al.*; 2004). Therefore, the physiological significance *in vivo* of any basal activity of endogenous A_{2A}AR expressed at low level is unclear. Gene dosage studies have provided evidence to show that, at least in T-lymphocytes, there is no A_{2A}AR reserve (Armstrong *et al.*; 2001). Consequently, pathophysiological conditions that alter A_{2A}AR expression, such as the onset of hypoxia (Kobayashi and

Millhorn; 1999) and EC exposure to Th1 cytokines (Nguyen *et al.*; 2003), are likely to alter cellular responsiveness to inflammatory stimuli. The availability of A_{2A}AR-deficient mice can now allow assessment of the presence and importance of A_{2A}AR expression and function as a potent anti-inflammatory mechanism *in vivo*. The effect of A_{2A}AR overexpression on STAT1 and STAT3 in this study was limited to HUVECs *in vitro*. Differences between endothelial types are well documented such as different vascular beds displaying distinct biochemical and immunological properties (Sands and Palmer; 2005). Arterial ECs for example are more sensitive to eicosanoids and less competent than venous and post capilliary ECs in their ability to upregulate adhesion molecule expression such as E-selectin and VCAM-1 (Amberger *et al.*; 1997).

The effect of mechanotrasduction has also not been investgated in this study. Normally ECs are exposed to various degrees of pulsatile and shear stresses that vary in magnitude in a manner determined by position along the vascular tree and local vascular tone. Such forces are well known to affect EC gene expression (Li *et al.*; 2005). So, the response *in vivo* may be different to those of culture system. Viral proteins could also change the endothelial function. Although experiments do use control virus it cannot rule out the possibility that viral protein somehow contribute to the observed effect of A_{2A}AR overexpression. However uninfected cells of very low passage (which still contain endogenous low levels of A_{2A}AR) still show inhibition of IL-6-mediated STAT phosphorylation when treated with an A_{2A}AR-selective agonist CGS21680 (W.Sands unpublished data).

Chapter 4

Mechanisms Controlling the Effects of A_{2A}AR Expression on Cytokine-Stimulated Degradation of STATs

4.1 Introduction

Physiological responses to cytokine stimulation must be regulated appropriately in order to prevent over active and damaging inflammatory processes. Several different mechanisms by which cytokine signalling is attenuated have been identified. The details, however, in determining the functions of specific inhibitors of cytokine signalling within particular cytokine signal transduction pathways have often been difficult to elucidate. SOCS knockout mice have pointed to the crucial functions of specific SOCS proteins in attenuating signalling by specific cytokines (Section 1.6). However, for the vast number of cytokines, the picture is complicated and in many cases multiple functionally redundant inhibitors are probably responsible for attenuating signal transduction. Identifying the roles of negative regulators of cytokine signalling in the plethora of signalling networks that are activated in response to cytokine is an interesting challenge.

The potent anti-inflammatory effect of the A_{2A}ARs have been demonstrated in a variety of different cell types (Section 1.5.1.1). One possible reason for this is that A_{2A}AR activation can activate common mechanisms that lead to the suppression of inflammation. The results in Chapters 3 and 4 suggest that the A_{2A}AR overexpression primes cytokine-activated STATs for degradation by the proteasome. The cytokine dependence of STAT degradation in A_{2A}AR-overexpressing cells raised the possibility that JAK-mediated STAT phosphorylation may responsible for this phenomenon. In addition, STATs may be ubiquitylated in A_{2A}AR-overexpressing HUVECs following cytokine exposure. Among the key proteins degraded by the Ub-proteasome system are those involved in the control of inflammation, cell cycle regulation and gene expression (Popat *et al.*; 2006). E3 Ub ligases play a key role in governing the cascade of Ub transfer reactions by recognising and catalysing Ub conjugation to specific protein substrates (Section 1.7). The E3s, which can be generally classified into HECT-type and RING-type families, are involved in the regulation of many aspects of the immune system, including the development,

activation, and differentiation of lymphocytes, induction of T cell-tolerance antigen presentation, immune evasion, and virus budding (Mueller; 2004). In addition to proteasome-mediated degradation, E3-promoted ubiquitination affects a wide array of biological processes, such as receptor down-regulation, signal transduction, protein processing or translocation, protein-protein interaction and gene transcription (Passmore and Barford; 2004). Deficiency or mutation of some of E3s, such as Cbl, Cbl-b or Itch, cause abnormal immune responses which can result in autoimmunity, malignancy, and inflammation (Bachmaier *et al.*; 2000; Wohlfert *et al.*; 2006).

Interestingly, IFN-γ is a key pleiotropic regulatory cytokine (Goldberg *et al.*; 2002). It controls an inducible proteolytic cascade, which consists of PA28 and other inducible proteasome subunits, and the activity of aminopeptidases, which lead to increase peptide production for MHC I presentation. In addition, IFN-γ also decreases peptide destruction by down-regulating the expression of a metalloproteinase, thimet oligopeptidase, that actively destroys many antigenic peptides (York *et al.*; 1999; Saveanu *et al.*; 2005). The UPS plays a significant role in the regulation of both T cell receptor (TCR) and co-stimulatory CD28 signalling through the action of ubiquitin ligases of the Cbl family (Zhang *et al.*; 2002a). CD28 co-stimulation results in the ubiquitination and degradation of Cbl-β, which eliminates the negative regulators and allows the expression of pro-inflammatory cytokines and their receptors. However, the most important link between the UPS and inflammation is related to NF-κB. NF-κB is a master regulator of many inflammatory cytokine genes, and its activation is mediated through the UPS. NF-κB is actively inhibited when bound to IκBα. NF-κB activation follows the degradation of IκBα, which is dependent on Lys 48-linked polyubiquination of IκBα followed by proteasomal degradation. In addition, the proteasome has been shown to regulate inflammatory responses by controlling the function of macrophages (Qureshi *et al.*; 2005). It has also been reported that the de-ubiquitinating enzyme CYLD posatively regulates proximal T cell receptor signalling in thymocytes by selectively binding to and deubiquitinating the active form of the kinase Lck. Due to defects in T cell development, CYLD-deficient mice had

substantially fewer mature CD4+ and CD8+ single-posative thymocytes and peripheral T cells (Reiley *et al.*; 2006). Hence, alterations in the UPS have profound effects on immune responses including the regulation of an array of inflammatory cytokines.

Results in Chapter 3 show that the A_{2A}AR overexpression primes cytokine-activated STATs for degradation by the proteasome. In addition, pre-incubation with proteasome inhibitor MG132 was sufficient to abolish the effect of the A_{2A}AR overexpression on priming STAT3 and STAT1 for down-regulation in response to cytokines. Because of that, we have investigated the responsibility of JAK for the tyrosine phosphorylation of STAT upon cytokine stimulation. In addition, we have tested the hypothesis that STAT is ubiquitylated in A_{2A}AR-overexpressing cells following cytokine stimulation before degradation by the proteasomal system.

4.2 Results

Phosphorylation is a commonly utilised signal that regulate substrate recognition by E3 ubiquitin ligases. For example, IκB kinase (IKK)-stimulated phosphorylation of IκBα on Ser32 and 33 is required for its recognition by the F-box component of the βTrCP/SCF1 E3 ligase complex (Liu *et al.*; 2005; Nalepa *et al.*; 2006). The cytokine dependence of STAT degradation in A_{2A}AR-overexpressing cells raised the possibility that JAK-mediated STAT phosphorylation was a trigger of this event. Thus, several experimental approaches were used to test this hypothesis in more detail. Firstly, the effect of inhibiting JAK activity on STAT degradation in A_{2A}AR-overexpressing cells was examined. This demonstrated that 0.1 μM of JAK inhibitor 1 (A potent cell-permeabile and ATP-competitive inhibitor of JAKs. Display spotent inhibitory activity against JAK1, JAK2, JAK3 and Tyk2) (Pedranzini *et al.*; 2006) was sufficient to inhibit JAK1 and JAK2 tyr-phosphorylation (Figure 4.1A) and STAT1 Tyr-phosphorylation (Figure 4.1B) upon sIL-6Rα/IL-6 stimulation. The same concentration of JAK inhibitor was sufficient to abolish the down-regulation of

STAT1 and STAT3 upon sIL-6Rα/IL-6 and IFNα stimulation in A_{2A}AR-overexpressing cells (Figure 4.2-4.5). Secondly, experiments utilised the presence in HUVECs of endogenous Ob-Rb leptin receptors. Leptin-bound Ob-Rb selectively stimulates JAK-mediated phosphorylation of STAT3 but not STAT1 (Zabeau *et al.*; 2003). Consistent with this observation, exposure of HUVECs to leptin specifically promoted the Tyr phosphorylation of STAT3 but not the Tyr phosphorylation of STAT1 whereas sIL-6Rα/IL-6, which was used as a positive control, promoted the Tyr phosphorylation of both STAT1 and STAT3 (Figure 4.6). In GFP-overexpressing cells, treatment with leptin produced phosphorylation of STAT3 on Tyr705. Overexpression of the A_{2A}AR significantly reduced STAT3 phosphorylation at each time point after 15 min (Figure 4.7). Leptin treatment also induced a time-dependent down-regulation of STAT3 but not STAT1 in A_{2A}AR-overexpressing cells after 1hr (Figure 4.8). Cytokine binding to its receptor leads to activation of the receptor-associated tyrosine kinase JAKs. JAKs trans-phosphorylate the intracellular domain of the receptor, and these phospho-Tyr residues provide docking sites for latent cytoplasmic STATs. STATs are recruited to the receptor via their SH2 domain, and JAKs phosphorylate STATs on a specific Tyr residue on their cytoplasmic tail. Homo- or heterodimerisation of STATs are achieved *via* reciprocal binding of this critical phospho-Tyr of one monomer and SH2 domain of the binding partner (Haan *et al.*; 1999). Following on from our results which show that the A_{2A}AR-overexpression can prime Tyr-phosphorylated STATs for degradation by the proteasome required JAK activity. There are two possible explanations for this requirement based on how JAK function. The first one is JAK Tyr-phosphorylated STAT. The second possibility is JAK Tyr-phosphrylation of the receptor. To discriminate between the two possibilities HUVECs were co-infected with AVs encoding the A_{2A}AR and either Flag epitope-tagged WT or Tyr705→Phe mutated STAT3, since mutation of Tyr705 renders STAT3 resistant to phosphorylation by JAKs (Kaptein *et al.*; 1996). Under conditions in which WT STAT3 underwent down-regulation in response to IFNα exposure similar to the effect observed for endogenous STATs, the levels of Tyr705→Phe mutated STAT3 were not altered

(Figure 4.9). Thus, JAK-mediated phosphorylation of STATs appears to be essential for promoting their cytokine-mediated degradation in A_{2A}AR-overexpressing HUVECs. Recently, SLIM/Mystique, a ubiquitin E3 ligase for Tyr-phosphorylated STATs was identified that promoted the polyubiquitination and degradation of phosphorylated STAT1 and STAT4 (Tanaka *et al.*; 2005). RT-PCR techniques were used to test for the presence of SLIM/Mystique mRNA in A_{2A}AR-overexpressing HUVECs (Figure 4.10). GAPDH was used as a control for the reverse transcription reaction and pcDNAA3/HA-SLIM/Mystique was included as a positive control for the SLIM/Mystique PCR reaction. The results showed that SLIM/Mystique was undetectable in our system, despite being able to detect the positive control. To confirm this lack of SLIM/Mystique mRNA in HUVEC was manifest at the protein level, I looked for the presence of SLIM/Mystique protein in HUVECs by western blot analysis. HUVECs treated as indicated in the figure legend were solubilised and analysed for expression of SLIM/Mystique protein by immunoblotting with anti-SLIM/Mystique antibody (Figure 4.11). Lysates from HEK293 cells transfected with either empty vector or a SLIM/Mystique expression construct were used as negative and positive controls respectively for the antibody reactivity (Figure 4.11). Together, these results suggest SLIM/Mystique is not expressed at detectable level under conditions in which STAT degradation occurs (Figure 4.11).

Cytokine activation of STATs is also negatively regulated by SOCS proteins, which can function as E3 ligases. In addition cAMP can induce SOCS3 via activation of Epac/Rap1 (Sands *et al.*; 2006). Thus, A_{2A}AR-overexpression may potentialy up-regulate SOCS3 expression upon cAMP activation leading to accelerated STAT degradation. To further identify the role of A_{2A}AR-overexpression in priming cytokine-activated STATs for degradation by the proteasome, the levels of SOCS3 protein was determined by immunoblotting and probing with an anti-SOCS3 antibody. The results showed that there is a siginficant difference in SOCS3 expression between GFP and A_{2A}AR-overexpressing cells upon cytokine stimulation at 2 and 3 hrs (Figure 4.12). However, this does not rule out the physiological

function of SOCS3 or other SOCS family members in the down-regulation of STAT1 and STAT3. Proteins targeted for proteasomal degradation are typically tagged on one or more Lys residues with Lys48-conjugated polyubiquitin chains (Liu *et al.*; 2005; Nalepa *et al.*; 2006). These chains are recognised as a degradation signal by the 26S proteasome, which then breaks down the target protein into its constituent amino acids for use in new protein synthesis and recycles polyubiquitin chains into monomers for further rounds of conjugation (Liu *et al.*; 2005; Nalepa *et al.*; 2006). To assess directly whether STATs were ubiquitylated in A_{2A}AR-overexpressing HUVECs following cytokine exposure, STAT3 was immunoprecipitated following denaturing cell lysis to remove any STAT-associated proteins and inactivate deubiquitylating enzymes (DUBs). Immunoblotting of STAT3 immunoprecipitates with anti-ubiquitin antibody revealed that HUVECs treatment with sIL-6Rα/IL-6 only resulted in the accumulation of a smear of ubiquitylated STAT3 in A_{2A}AR-overexpressing cells (Figure 4.13). Thus, the accumulation of ubiquitin-conjugated STATs in HUVECs occurred under conditions that also promoted their degradation.

A similar immunoprecipitation approach was attempted for STAT1 but was unsuccessful due to its inefficient immunoprecipitation with commercially available antibodies. Instead, a strategy employing the ability of the UBA domain from *Saccharomyces cerevisiae* protein Dsk2p to specifically isolate ubiquitylated proteins from whole cell extracts *in vitro* was used (Funakoshi *et al.*; 2002). Using recombinant GST-Dsk2pUBA immobilised to glutathione-Sepharose, Tyr701-phosphorylated STAT1 could only be captured from A_{2A}AR-overexpressing HUVECs after treatment with sIL-6Rα/IL-6, i.e. conditions that promoted STAT degradation (Figure 4.14). Probing of immunoblots with an anti-ubiquitin antibody also confirmed the ability of GST-Dsk2pUBA but not GST alone to specifically capture ubiquitylated proteins from soluble HUVECs extract (Figure 4.14).

Ubiquitin is a relatively stable protein in yeast despite its covalent linkage to many proteins destined for proteasomal or vacuolar degradation (Swaminathan *et al.*;

1999). This is possible because ubiquitin-protein modification is transient. Deubiquitylating enzymes release ubiquitin from polyubiquitin conjugates by cleaving the isopeptide bond between the ubiquitins in a chain or at the ubiquitin C terminus linked to substrate. The yeast DUB family consists of at least 20 members, including 16 in the ubiquitin-specific processing protease (UBP) subfamily (Amerik *et al.*; 2000; Amerik and Hochstrasser; 2004). To further confirm the specificity of STAT binding to GST-Dsk2pUBA *in vitro*, immobilised proteins were incubated with or without recombinant human isopeptidase T/UBP5, which selectively removes ubiquitin monomers from modified proteins (Lacombe and Gabriel; 2002). Under these conditions, incubation of GST-DsK2pUBA-immobilised beads with UBP5 promoted the release of Tyr701-phosphorylated STAT1 (Figure 4.15). No significant release was detectable if the reaction was performed at 4 ^{o}C, suggesting that the release of STAT1 from GST-Dsk2pUBA required its deubiquitylation by enzymatically active UBP5 (Figure 4.15).

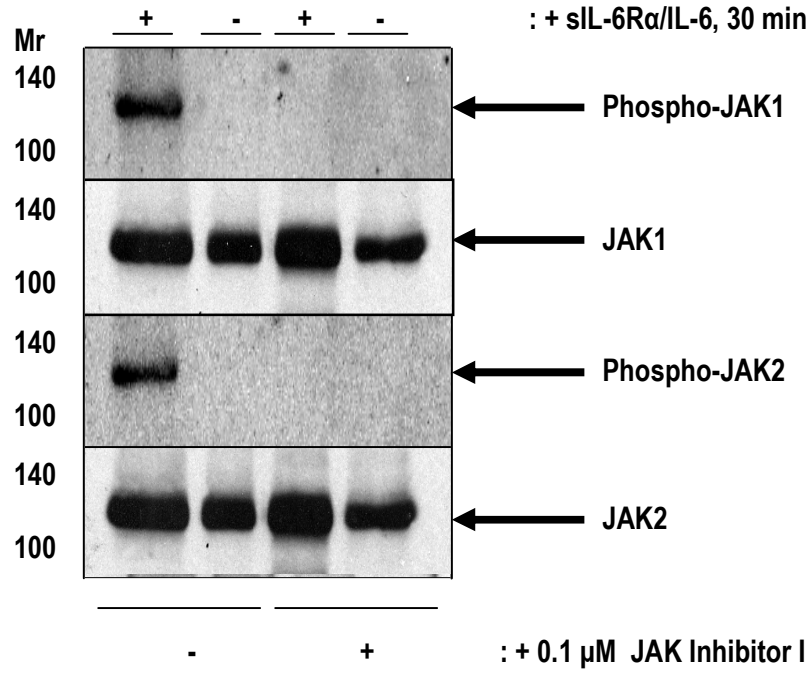
To determine the relationship between STAT ubiquitylation and Tyr phosphorylation in A_{2A}AR-overexpressing cells, Flag-tagged WT and Tyr705→Phe-mutated STAT3 were co-expressed in HUVECs with the A_{2A}AR and immunoprecipitated with anti-Flag antibody-conjugated Sepharose beads following denaturing cell lysis after cytokine treatment (Figure 4.16). This demonstrated that under conditions in which recombinant WT STAT3 is polyubiquitylated similarly to the endogenous STAT3 in response to sIL-6Rα/IL-6, no ubiquitylation of the Tyr705→Phe-mutated STAT3 could be detected (Figure 4.16). Taken together, these data are consistent with a model where by A_{2A}AR-overexpression specifically primes JAK-phosphorylated STATs for polyubiquitylation and subsequent degradation by the proteasome.

Figure 4.1 Effect of JAK inhibition 1 on sIL-6Rα/IL-6-induced JAK1, JAK2 and STAT1 phosphorylation

Panel A: $1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without sIL-6Rα/IL-6 for 30 min as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equlised for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-phospho-JAK1, anti-phospho-JAK2, anti-JAK1 and anti-JAK2.

Panel B: $1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without sIL-6Rα/IL-6 for 30 min as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equlised for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-phospho-STAT1 antibody.

A


+ - + -
: + sIL-6Rα/IL-6, 30 min
Mr
140
100
Phospho-JAK1
JAK1
Phospho-JAK2
JAK2
- +
: + 0.1 μM JAK Inhibitor I

B

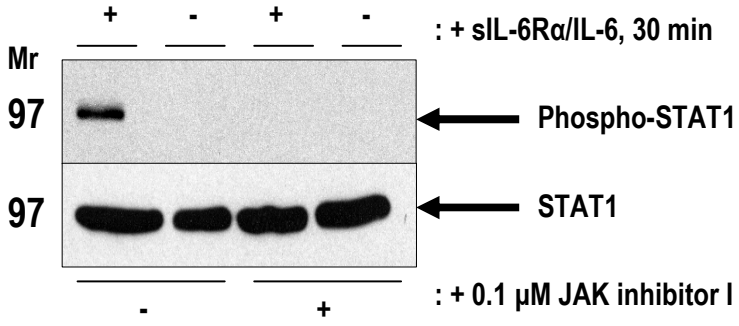

+ - + -
: + sIL-6Rα/IL-6, 30 min
Mr
97
Phospho-STAT1
STAT1
- +
: + 0.1 μM JAK inhibitor I

Figure 4.2 Effect of JAK inhibition on sIL-6Rα/IL-6-induced STAT1 phosphorylation and downregulation of STAT1 in A_{2A}AR expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 up to 3 hrs as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT1 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT1 levels in vehicle-pretreated cells at the given time point). Basal set at 100.

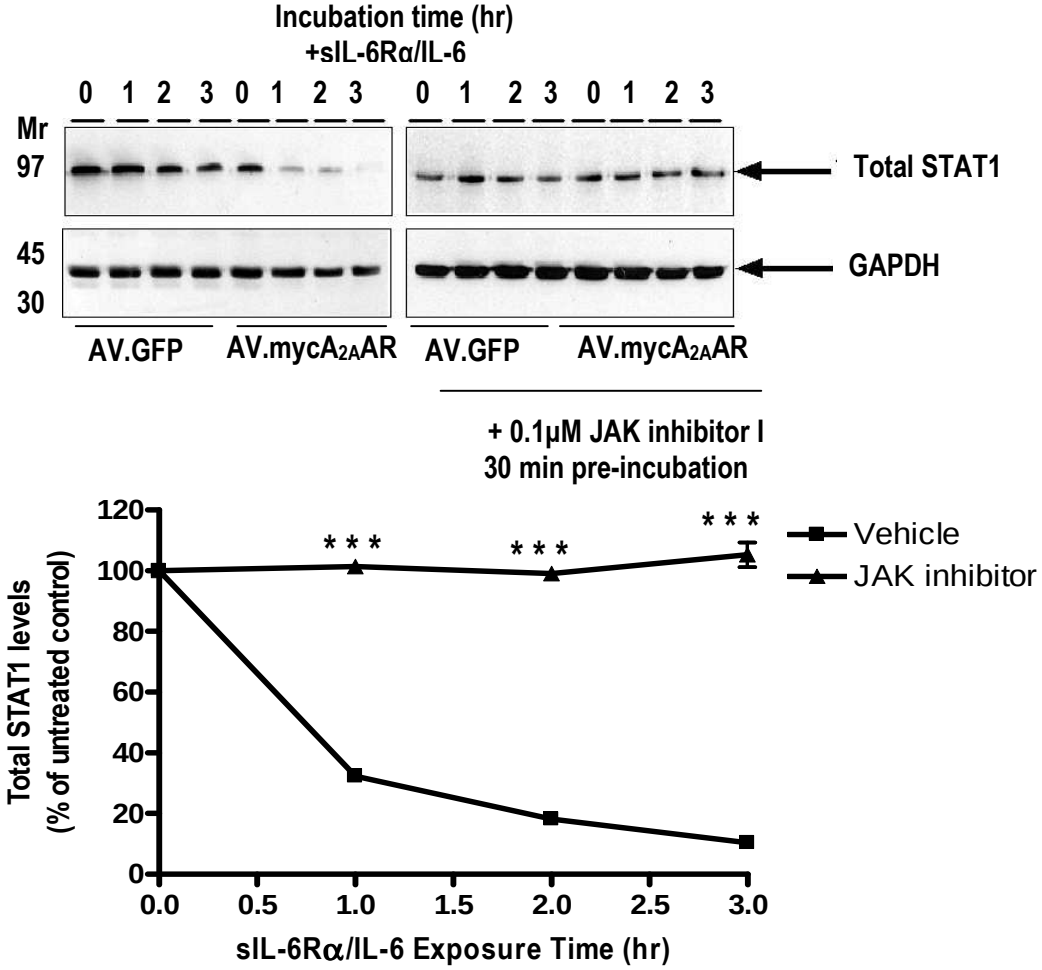


Figure 4. 3 Effect of JAK inhibition on sIL-6Rα/IL-6-induced STAT3 downregulation in A_{2A}AR expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 up to 3hr as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT3 levels in vehicle-pretreated cells at the given time point). Basal set at 100.

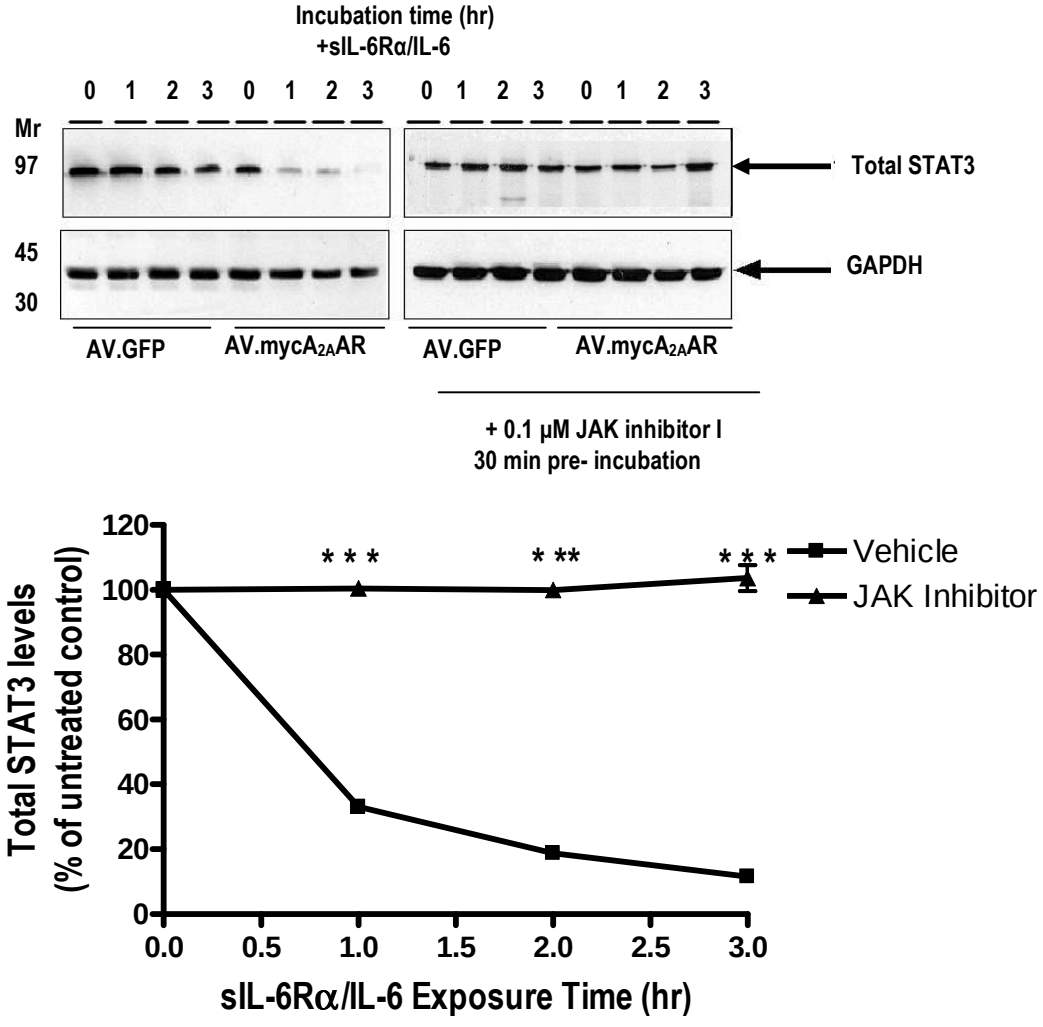


Figure 4.4 Effect of JAK inhibition on IFNα-induced STAT1 downregulation in A_{2A}AR expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without 500 unit/ml IFNα up to 3hr as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT1 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT1 levels in vehicle-pretreated cells at the given time point). Basal set at 100.

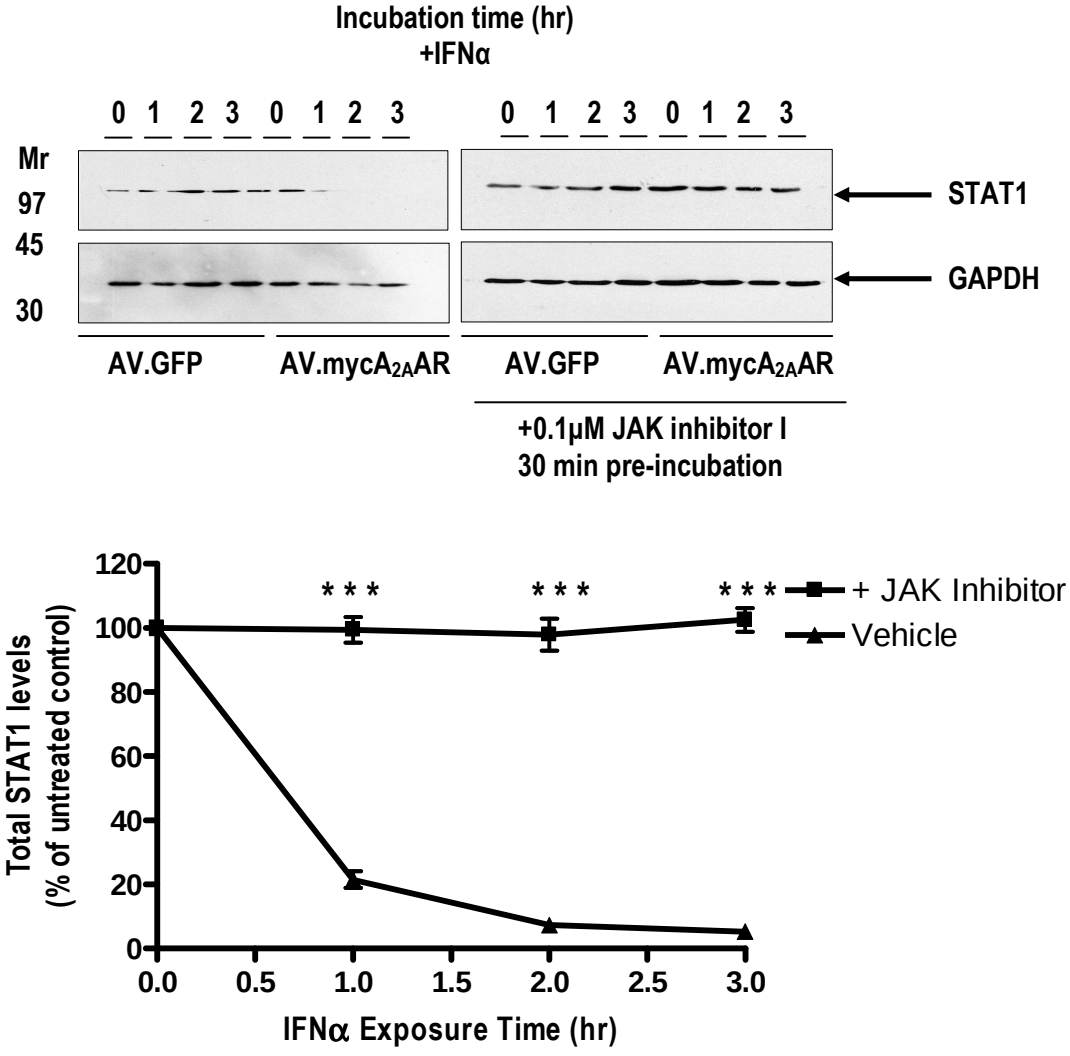


Figure 4.5 Effect of JAK inhibition on IFNα-induced STAT3 downregulation in A_{2A}AR expressing HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without 500 unit/ml IFNα up to 3hr as indicated. The final concentration of vehicle (DMSO) in all wells was 0.1% (V/V). Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three experiments is presented (***p<0.001 *versus* STAT3 levels in vehicle-pretreated cells at the given time point). Basal set at 100.

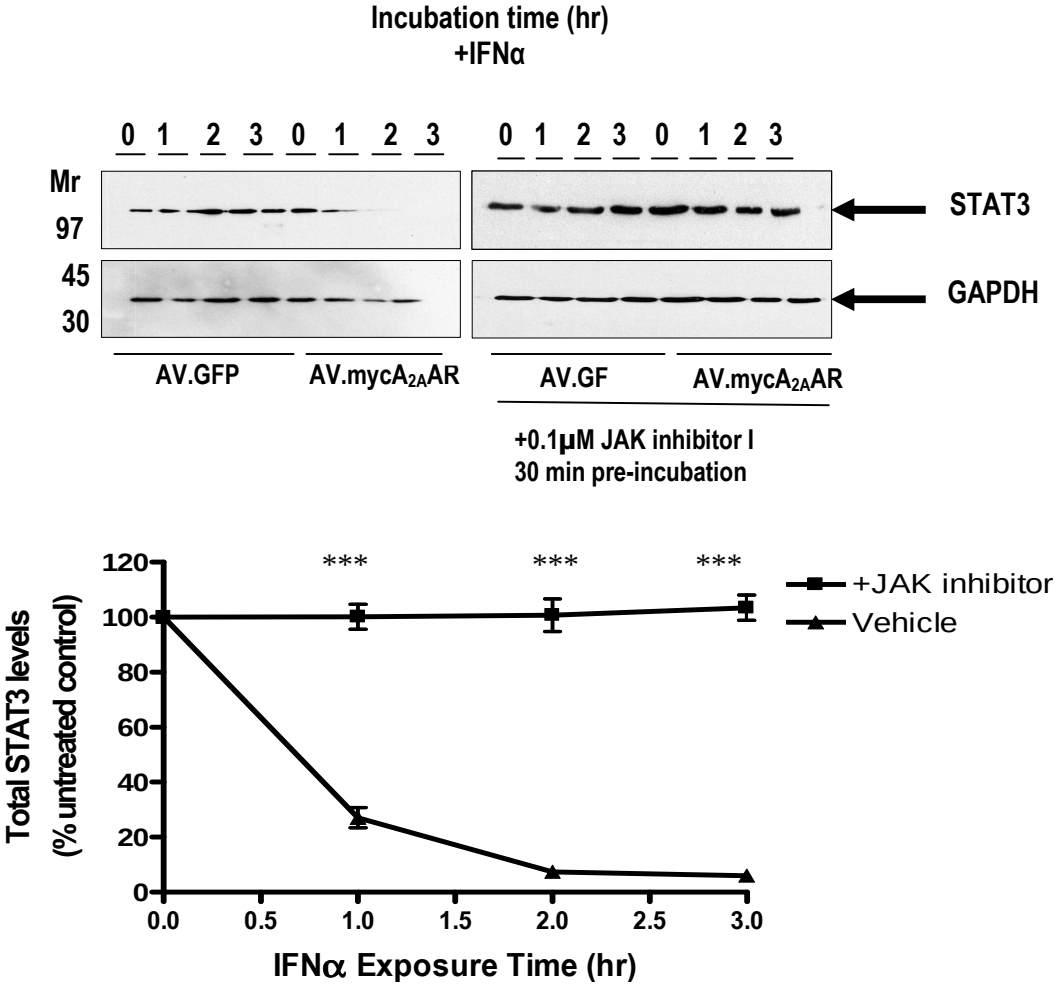


Figure 4.6 Leptin stimulates the specific phosphorylation of STAT3 but not STAT1

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 or 100 ng/ml leptin for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies.

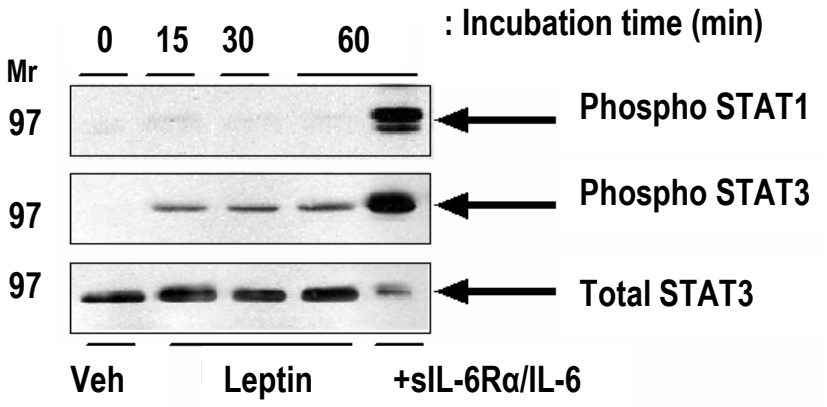


Figure 4.7 Effect of A_{2A}AR gene transfer on STAT3 phosphorylation and expression in response to leptin

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 100 ng/ml leptin for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phospho-STAT3 and STAT3 levels from three experiments is presented (**p<0.005 and *p<0.05 *versus* corresponding STAT levels in AV.GFP-infected cells at the given time point). Basal set at 100.

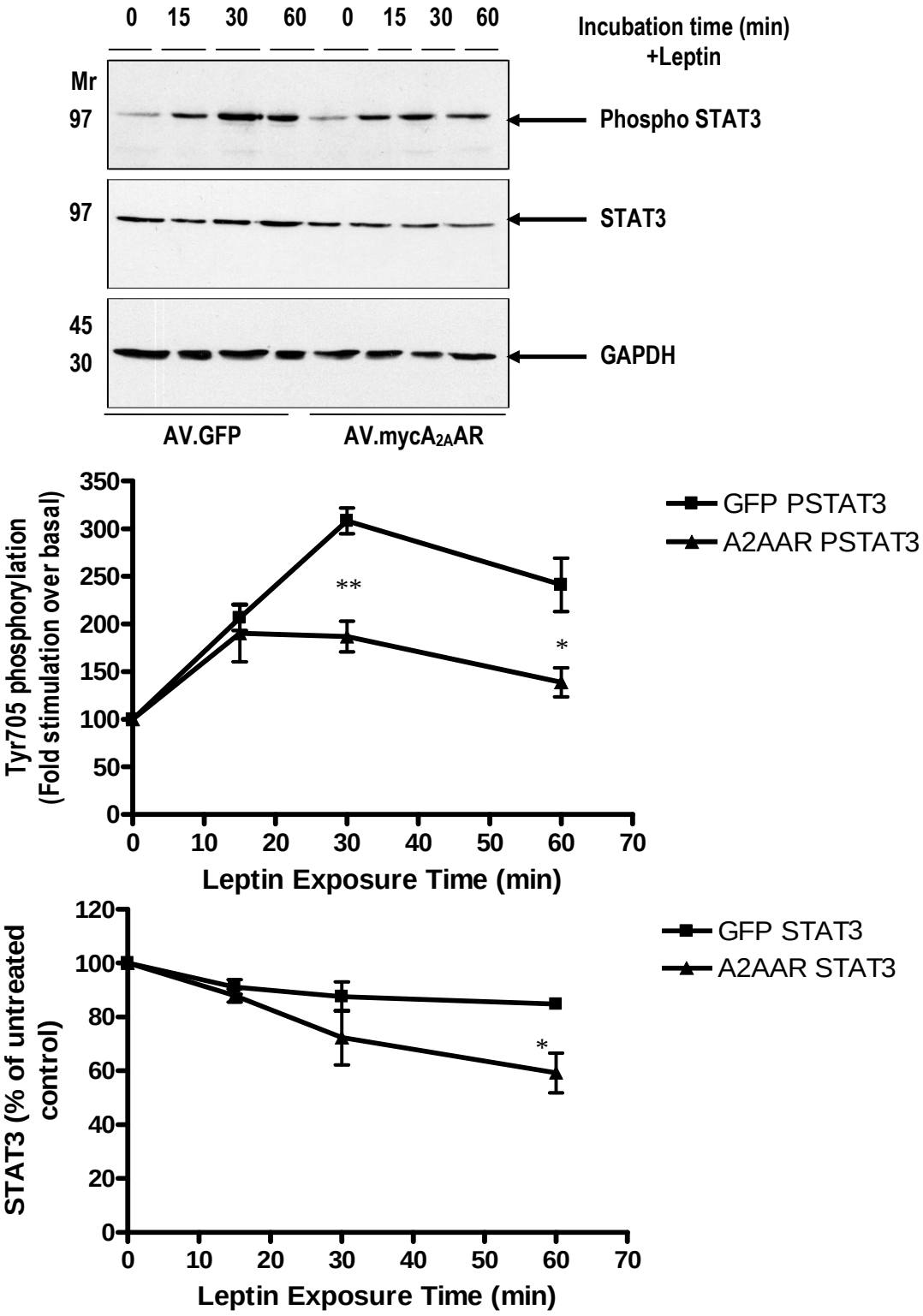
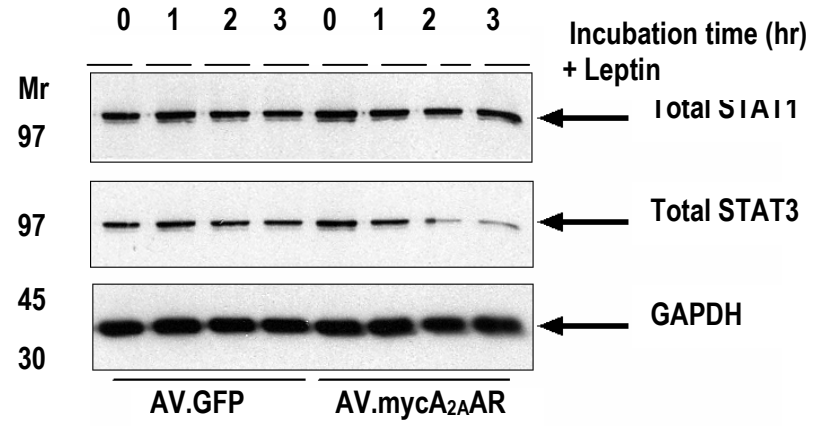
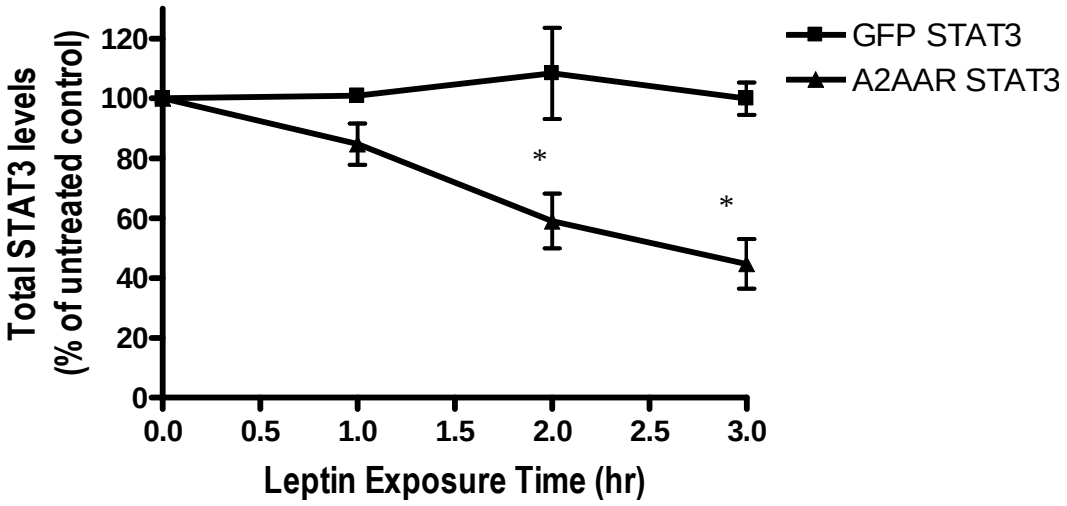

0 15 30 60 0 15 30 60
Incubation time (min)
+Leptin
Mr
97
Phospho STAT3
97
STAT3
45
30
GAPDH
AV.GFP
AV.mycA2AAR
Tyr705 phosphorylation
(Fold stimulation over basal)
GFP PSTAT3
A2AAR PSTAT3
**
*
Leptin Exposure Time (min)
STAT3 (% of untreated control)
GFP STAT3
A2AAR STAT3
*
Leptin Exposure Time (min)

Figure 4.8 Effect of A_{2A}AR gene transfer on STAT1 and STAT3 expression in response to prolonged exposure to leptin

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 100 ng/ml leptin for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three experiments is presented (*p<0.05 versus corresponding STAT levels in AV.GFP-infected cells at the given time point). Basal set at 100.


0 1 2 3 0 1 2 3
Incubation time (hr)
+ Leptin
Mr
97
Total STAT1
97
Total STAT3
45
30
GAPDH
AV.GFP
AV.mycA2AAR


Total STAT3 levels
(% of untreated control)
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Leptin Exposure Time (hr)
GFP STAT3
A2AAR STAT3
*
*

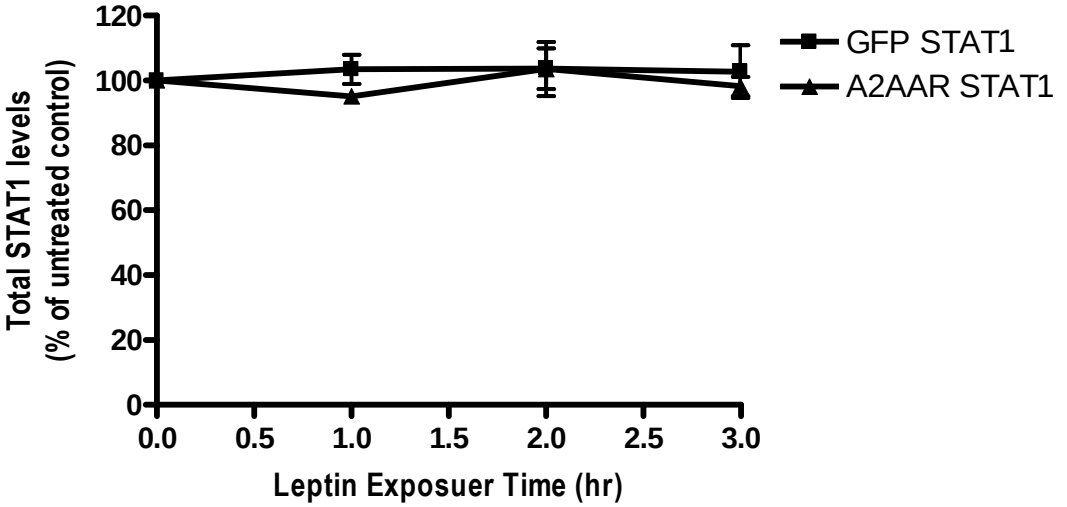

Total STAT1 levels
(% of untreated control)
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Leptin Exposuer Time (hr)
GFP STAT1
A2AAR STAT1

Figure 4.9 Effect A_{2A}AR gene transfer on WT and Tyr705→Phe mutated STAT3 expression levels.

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were co-infected with AV myc-A_{2A}AR and either AV. Flag-WT STAT3 or AV.Flag-Tyr705→Phe mutated STAT3 prior to treatment with or without 500 unit/ml IFNα for 1 hr as indicated. Soluble cell exracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of WT and Tyr705→Phe mutated STAT3 down-regulation in A_{2A}AR-expressing cells from three experiments is presented (***P <0.001 versus WT). Basal set at 100.

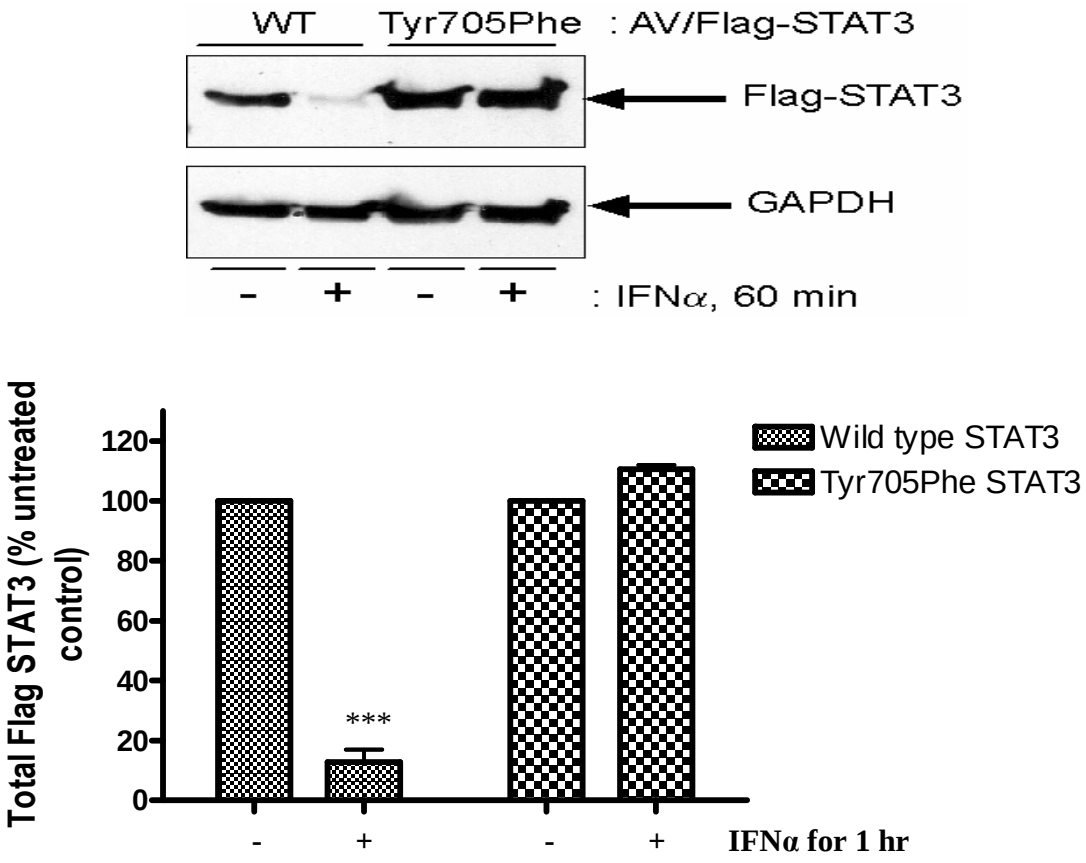


Figure 4.10 Effect of A_{2A}AR gene transfer on mRNA levels of the phospho STAT-specific E3 Ub ligase SLIM/Mystique in vascular endothelial cells under conditions that promote STAT degradation

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 2hr. Cells were harvested into 0.5 ml of Tri-reagent and RNA extracted as described in section 2.3.6. 1μg RNA of each sample was then used to generate cDNA as described in 2.3.7. To assess SLIM/Mystique expression 1 μl of cDNA was used per reaction. As positive control 50 ng of pcDNAA3/HA-SLIM/Mystique plasmid was used. PCR conditions were given in section 2.3.8. DNA was then fractionated by agarose gel. Results are typical of three experiments is presented.

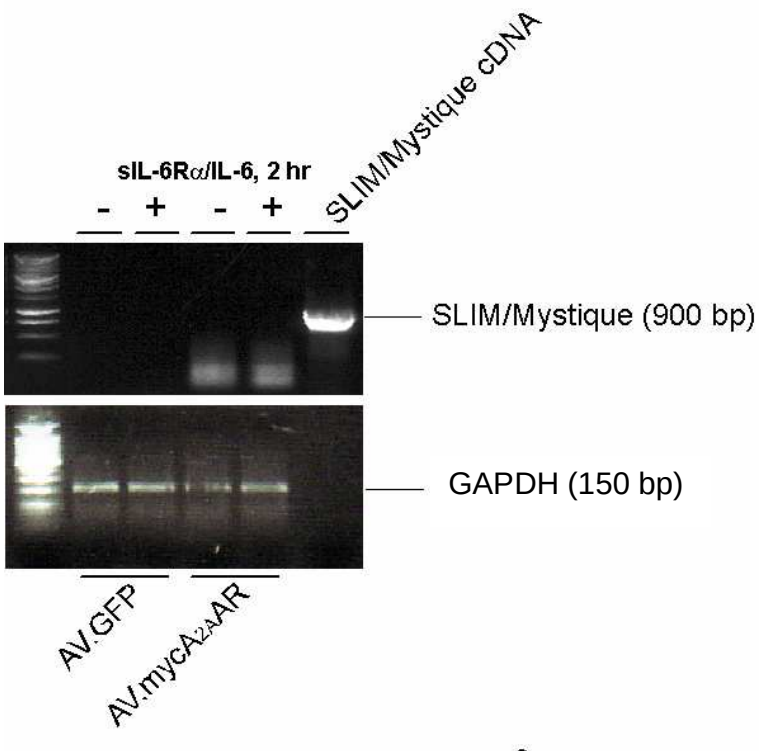


Figure 4.11 Effect of A_{2A}AR gene transfer on protein expression of the phospho STAT-specific E3 Ub ligase SLIM/Mystique in vascular endothelial cells under conditions that promote STAT degradation

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 2hr. Soluble cell extract of HEK293 cells express pcDNAA3/HA-SLIM/Mystique plasmid used as positive control. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies.

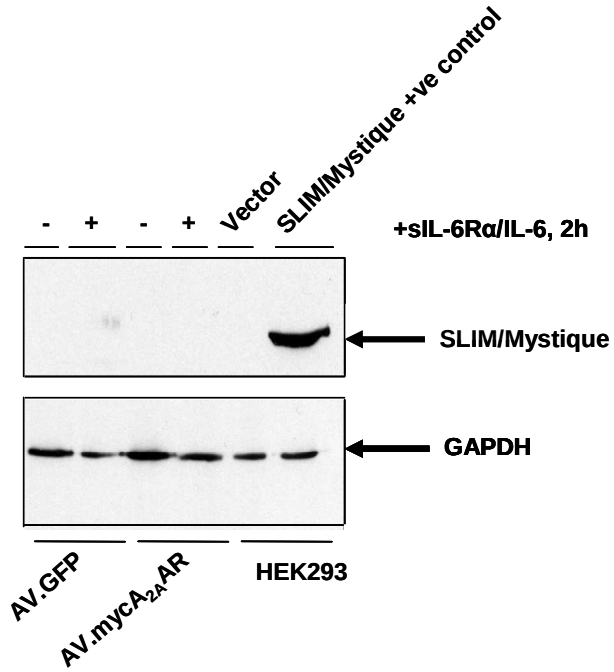
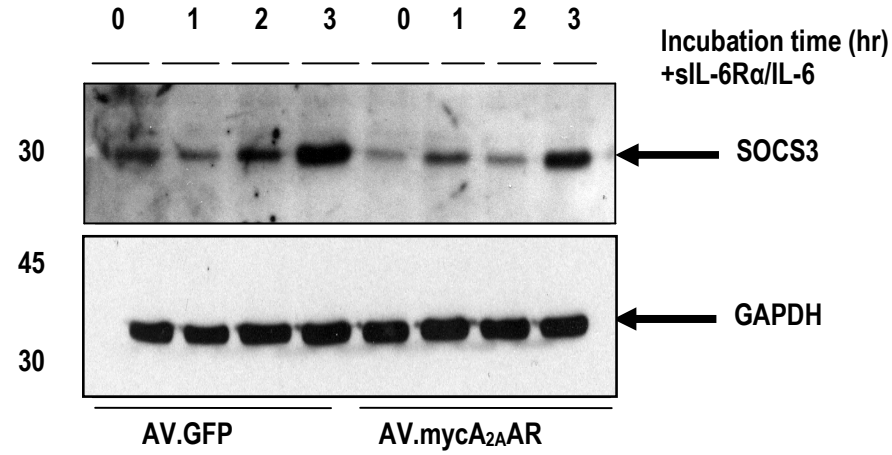


Figure 4.12 Effect of A_{2A}AR gene expression on SOCS3 induction in response to sIL-6 Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with MG132 for 30 min and treatment with 25ng/ml sIL-6 Rα/5ng/ml IL-6 for up to 3 hrs as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels from three experiments is presented. Control 100 set at 0 hr of GFP cells.



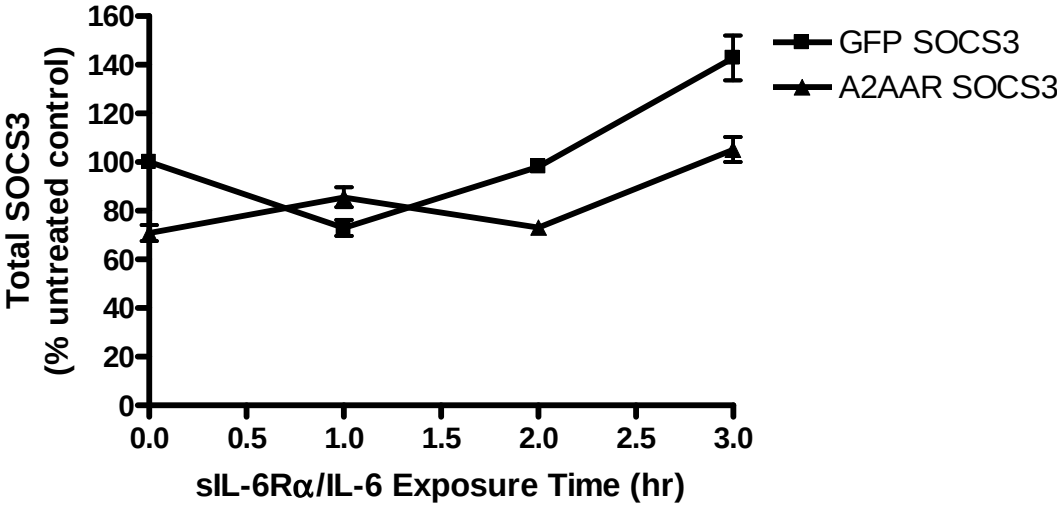


Figure 4.13 A_{2A}AR expression primes STAT3 for cytokine-triggered ubiquitylation

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to pre-treatment with or without 6μM MG132 for 30 min and incubation with 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 1 hr. Samples were then denatured by heating in SDS-containing buffer prior to dilution into excess non-ionic detergent for preparation of clarified extracts and immunoprecipitation of STAT3. Immunoprecipitates were fractionated by SDS-PAGE for immunoblotting with anti-ubiquitin and STAT3 antibodies.

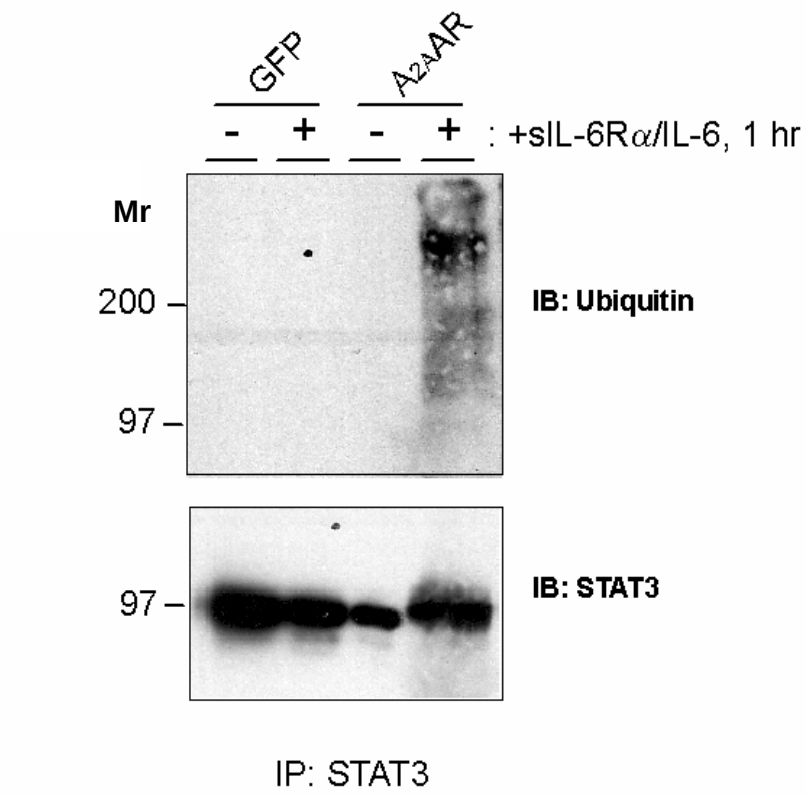


Figure 4.14 A_{2A}AR expression primes STAT1 for cytokine-triggered ubiquitylation

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with AV.mycA_{2A}AR were pre-treated with 6μM MG132 followed by exposure to 25ng/ml sIL-6 Rα/5ng/ml IL-6.Cells were then harvested for preparation of protein-equalised soluble fractions and pull-down experiment using 5ug/sample of GST (negative control) or GST-Dsk2pUBA. Captured proteins were fractionated by SDS-PAGE for immunoblotting with anti-ubiquitin, phospho-STAT1 and total STAT1 antibodies. This is one of three experiments that produced similar results.

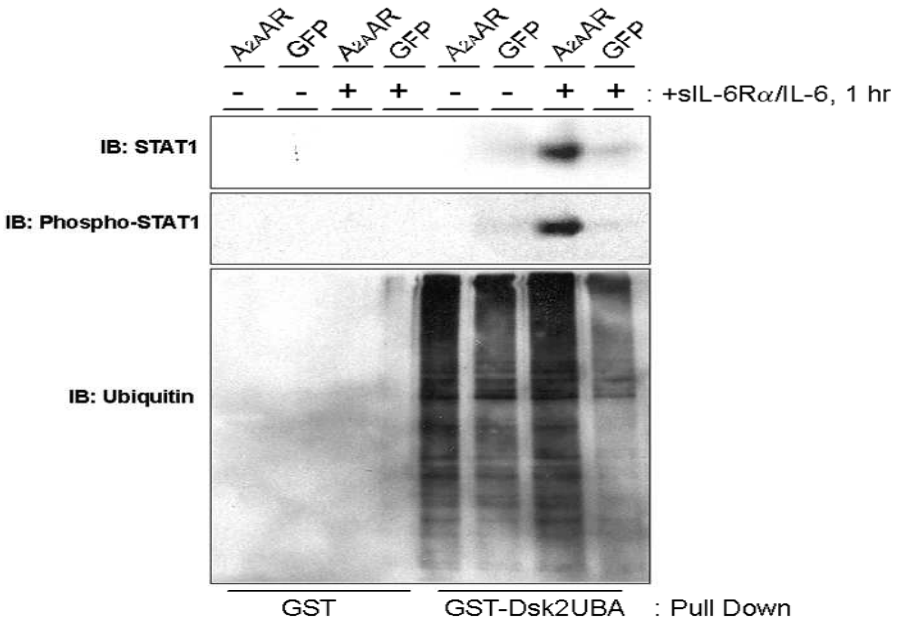


Figure 4.15 Effect of UBP5 on release of STAT1 from GST-Dsk2p

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with AV.mycA_{2A}AR were pre-treated with 6μM MG132 followed by exposure to 25ng/ml sIL-6Rα/5ng/ml IL-6. Cells were then harvested for preparation of protein-equalised soluble fractions and capture of ubiquitylated proteins with GST-Dsk2pUBA. Following the final wash, beads were resuspended in reaction buffer and incubationed with or without UBP5 for 1hr at the indicated temperatures. The reaction buffer was then analysed for the presence of Tyr701-phosphorylated and total STAT1 by SDS-PAGE and immunoblotting. This is one of three experiments that produced similar results.

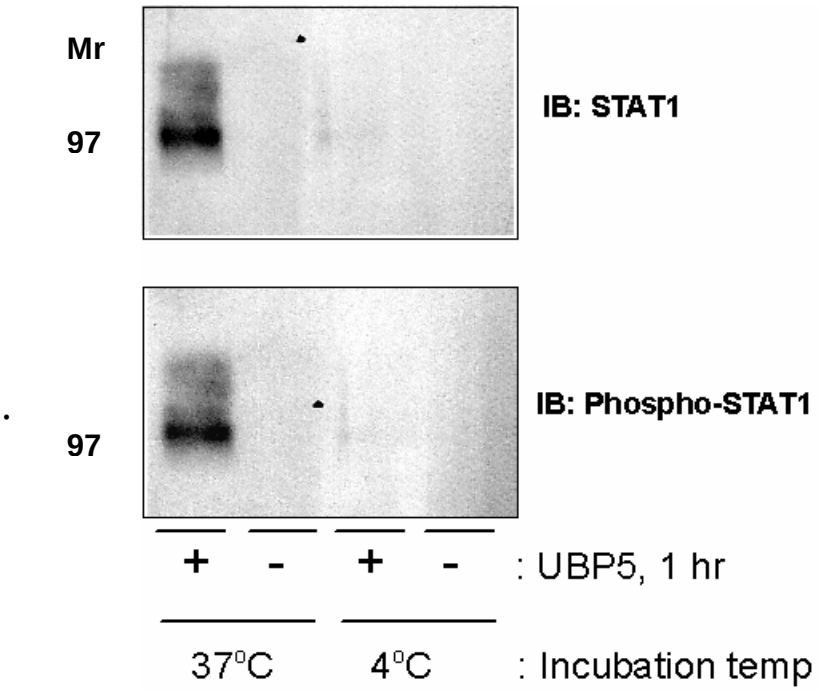
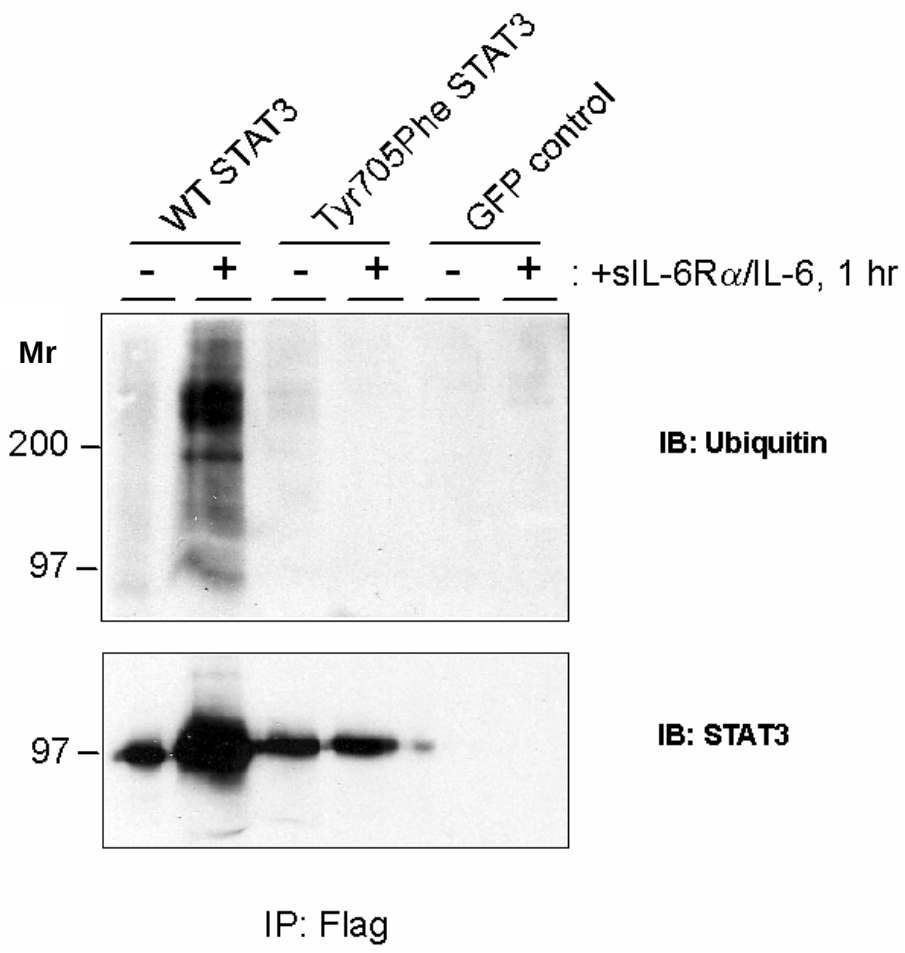


Figure 4.16 A_{2A}AR expression primes Flag-WT but not Tyr705→Phe mutated STAT3 for cytokine-triggered ubiquitylation

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were co-infected with AV.myc-A_{2A}AR and either AV.Flag-WT STAT3 or AV.Flag-Tyr705→Phe mutated STAT3 pritotreatment with 6μM MG132 and 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 1 hr as indicated. Cells were then denatured by heating in SDS-containing buffer prior to dilution into excess non-ionic detergent for preparation of clarified extracts and immunoprecipitation of Flag-STAT3 proteins using M2 antibody-coupled Sepharose beads. Samples were then fractionated by SDS-PAGE prior to immunoblotting with anti-ubiquitin and STAT3 antibodies.



4.3 Discussion

JAKs are no longer implicated only in classic cytokine receptor-mediated signalling pathways, but are now also known to integrate indirectly into other receptor-mediated signal transduction processes. Therefore, an increasing number of therapeutic applications exist for biological response modifiers that can modulate JAK/STAT activity. Exciting breakthroughs in both physiological and pharmacological methods of selective inhibition of cytokine JAK-STAT pathways have recently emerged in the form of suppressors of cytokine signalling. The basis of these and other mechanisms of negative regulation of JAK activity, including the suppression of JAK expression levels caused by tumour- or pathogen-derived agents, the complex interactions of JAKs with phosphatases. The possibility of modulating selected JAK/STAT-mediated cellular signals by inhibiting JAK kinase activity to obtain a posative therapeutic outcome is a tantalising prospect, as yet incompletely realised. While current data suggest no therapeutic use for JAK1 and TyK2 inhibition, JAK2 inhibition seems a promising but untested strategy for therapeutic intervention (Reiterer and Yen; 2006). More promising, however, are the data indicating a possible therapeutic use of JAK inhibition. Results in this Chapter showed that degradation of STATs required JAK activity since 1) it was blocked by pre-incubation with JAK inhibitor 1. 2) STAT1 but not STAT3 was resistant to both tyrosine phosphorylation and down-regulation in response to leptin and 3) a Tyr705→Phe mutated STAT3 was also resistant to cytokine-triggered degradation, suggesting that JAK-mediated phosphorylation of this residue is required to produce the effect. The importance of JAK in mediating cytokines signalling has been shown in many studies including *in vivo* studies, where Tyk2 has been shown to be partially necessary for IFN-α/β-induced tyrosine phosphorylation of STAT3 (Karaghiosoff *et al.*; 2000; Shimoda *et al.*; 2000). The SOCS protein family comprises a group of cytokine-inducible genes that were discovered initially to suppress STAT signalling by binding to and inhibiting JAKs (Starr and Hilton; 1999; Yasukawa *et al.*; 1999). Some of these proteins are transcriptionally regulated by STATs themselves,

suggesting that STATs can negatively regulate their own phosphorylation state. The kinase activity of the Tel-JAK2 fusion protein is associated with leukemia and known to activate STAT5 (Levy and Gilliland; 2000). SOCS1 has been demonstrated to block Tel-JAK2-mediated transformation of hematopoietic cells (Frantsve *et al.*; 2001). Recently, a deletion on chromosome 16p that contains SOCS1 has been found in 48% of primary hepatocellular carcinomas, raising the possibility that inactivation of this gene may participate in hepatocarcinogenesis (Koyama *et al.*; 1999). It can therefore be speculated that negative regulators of STAT signalling might play important roles in the control of tumour incidence and/or progression.

Many studies have demonstrated that GPCRs have been shown to be connected to the JAK/STAT pathway (Lukashova *et al.*; 2001). However, the mechanisms involved in regulating the activation of this signalling pathway by GPCRs remain limited. In addition, little is known about the role of the JAK pathway in the physiological or pathophysiological functions of GPCRs. Despite the obvious importance of the A_{2A}AR in controlling the expression and function of various transcription factors such as p53 (Watson and Irwin; 2006), NF-κB (Chen; 2005) and Smads (Izzi and Attisano; 2006), relatively few reports have examined Ub capacity to regulate STATs. Our results show that over-expression of the A_{2A}AR in HUVECs led to the more rapid degradation of STAT1 and STAT3 in A_{2A}AR overexpressing cells. Interestingly, cAMP-elevating agents have been shown to inhibit IL-6-induced STAT activation in monocytes (Sengupta *et al.*; 1996). These data establish a link for crosstalk between cAMP-mediated signalling pathway and STAT, and suggest that A_{2A}AR mediated inhibition of STAT may possibly be cAMP-dependent.

The ability of V proteins encoded by paramyxoviruses to function as STAT E3 ubiquitin ligases is a well-established mechanism by which they subvert the interferon response (Horvath; 2004a), native cellular mechanisms controlling STAT degradation are rather less well defined. The first description of STAT degradation centred around the observation that proteasome inhibition produced a more robust

accumulation of tyrosine-phosphorylated STAT1 in HeLa cells following exposure to IFNγ. This suggests that proteasomal degradation is an important mechanism by which STAT1 function is turned off in these cells (Kim and Maniatis; 1996). It was been shown that removal of IL-3 from 32D myeloid cells results in a time-dependent proteasomal degradation of STAT5 but not STAT1, 2 or 3 (Wang *et al.*; 2000), although nuclear translocation rather than tyrosine phosphorylation seems to be required for degradation to occur (Chen *et al.*; 2006). Another study has shown that the degradation of STAT3 in H4IIE hepatoma cells could be triggered by hyperosmotic stress and occurred independently of phosphorylation on Tyr705 (Schafer *et al.*; 2005). Taken together, none of these observations are consistent with a single unifying mechanism. Thus, while the recent identification of the protein "SLIM" as an E3 ubiquitin ligase able to trigger the polyubiquitylation of STAT1 and STAT4 is important (Tanaka *et al.*; 2005), it is unlikely to account for all STAT degradation phenomena reported in the literature. In relation to this issue it is important to note that inhibition of proteasome function was sufficient to block the inhibitory effect of the A_{2A}AR-overexpression on STAT3 phosphorylation (Figures 3.17-3.20). This demonstrates that priming of STATs for degradation is the only mechanism responsible for the reduced cytokine-stimulated STAT phosphorylation observed in A_{2A}AR overexpressing cells (see Chapter 3). Identification of Ub-conjugation states and nature of chain linkage on STAT3-recognising the states of Ub attachment and identification of the E3 ligase(s) responsible for STAT ubiquitylation would define new targets on STATs amenable to therapeutic manipulation. However, this identification is hampered by the lack of any consensus ubiquitylation sequences displayed by E3s (Nalepa *et al.*; 2006) and assuming the ubiquitylation states are conserved, the presence of more than 20 Lys residues common between human STATs 1 and 3. However, the results presented in my thesis suggest that there is no detectable SLIM message or protein under conditions in which STAT degradation occurs (Figure 4.10 and 4.11), suggesting that another E3 is responsible. Identification of this new E3 would constitute a major advance towards designing strategies for manipulating STAT function. It is becaming increasingly appreciated

that SOCS proteins also act by promoting the degradation of specific signalling proteins. Recent work suggests that SOCS box-containing proteins act as adapter molecules that recruit activated signalling proteins to the proteasome. The results showed that there is a siginficant difference in SOCS3 expression between GFP and A_{2A}AR overexpressing cells upon cytokines stimulation at 2 and 3 hrs (Figure 4.12). However, this does not rule out the physiological function of SOCS3 or other SOCS family members in the down-regulation of STAT1 and STAT3. In addition, we used WT and SOCS3 knockout mouse embryonic fibroblasts (MEFs) to test the inolvement of SOCS3 in STAT down-regulation. However, these experiments proved to be technically difficult as we could not transduce them with the adenovirues at our disposal. One study has reported that expression of SOCS1 but not SOCS3 promotes the degradation of ubiquitylated JAK2 (Shuai and Liu; 2003) leading to a reduction in STAT activation. In addition, STAT1 is known to be regulated by phosphorylation-dependent polyubiquitination in response to IFN-γ (Kim and Maniatis; 1996). Given that SOCS1 can promote the degradation of JAK2, it is also possible that SOCS1 may target the degradation of associated STAT1.

In addition, to examine the polyubiquitination of STATs, extracts from A_{2A}AR overexpressing cells treated or not treated with proteasome inhibitor MG132 were subjected to immunoprecipitation analysis with anti-STAT3 antibody or pull down experiments. It is obvious that the amounts of phosphorylated STAT in A_{2A}AR expressing cells upon sIL-6Rα/IL-6 stimulation are controlled by the ubiquitin-dependent proteolysis of STAT1 and STAT3, consistent with the finding that STAT1 proteins activated by IFN-γ may be negatively regulated by the ubiquitin-proteasome pathway (Kim and Maniatis; 1996). These results strongly suggest that STAT1 and STAT3 are polyubiquitinated before their degradation by the 26S proteasome. In addition, Tyr phosphorylation is required for cytokine-triggered degradation of STATs following A_{2A}AR overexpression. However, it is unclear where within the cells ubiquitylation and degradation occur, which residues on STATs confer

sensitivity to polyubiquitylation, and the nature of the polyubiquitylation chain linkage.

Finally, in this study we have demonstrated that the A_{2A}AR overexpression can prime cytokine-activated STATs for polyubiquitylation and subsequent degradation by the proteasome. It was found that overexpression of the A_{2A}AR in the absence of any agonist was sufficient to prime STATs for degradation. It is possible that over the course of the experiments, endogenous adenosine released by HUVECs *in vitro* reaches extracellular levels sufficient to cause A_{2A}AR activation. However signalling from cytokine receptors is subject to strict negative regulation via several mechanisms designed to prevent inappropriately sustained activation of downstream responses (Wormald and Hilton; 2004). In most systems activation of STAT is transient. This suggests that efficient mechanisms for STAT inactivation must exist. At least two possible mechanisms can be envisioned. The first one is dephosphorylation of Tyr-phosphorylated STAT. The second mechanism is degradation of Tyr-phosphorylated STAT. To determine whether the Tyr-phosphorylation of STAT1 and STAT3 is involved in ubiquitination and proteasome-mediated degradation, Flag-tagged WT STAT3 and Tyr705→Phe mutated STAT3 were used. This demonstrated that under conditions in which recombinant WT STAT3 is polyubiquitylated similarly to the endogenous STAT3 in response to sIL-6Rα/IL-6, no ubiquitylation of the Tyr705→Phe-mutated STAT3 could be detected. This result suggested that Tyr-phosphorylation of STAT was crucial for STAT ubiquitination and degradation in A_{2A}AR overexpressing cells, since we could not observed the same effect in Tyr705→Phe-mutated STAT3 as well in untreated cells with cytokines.

Phosphorylation is a commonly utilised signal involved in controlling substrate recognition by multiple ubiquitin E3 ligases. The cytokine dependence of STAT degradation in A_{2A}AR overexpressing cells raised the possibility that JAK-mediated STAT phosphorylation was a trigger for this event. Thus, several experimental approaches in Chapter 4 were used to test this hypothesis in more detail.

(1) JAK inhibitor 1 inhibits Tyr-phosphorylation of JAK1, JAK2 and STAT1.

(2) Degradation required JAK activity since;

a) It was blocked by pre-incubation with JAK inhibitor 1.

b) STAT1 but not STAT3 was resistant to both tyrosine phosphorylation and down-regulation in response to leptin.

c) A Tyr705→Phe mutated STAT3 was also resistant to cytokine-triggered degradation, suggesting that JAK-mediated phosphorylation of this residue is required to produce the effect.

(3) To assess directly whether STATs were ubiquitylated in A_{2A}AR overexpressing HUVECs following cytokine exposure, STAT3 and Flag-tagged WT STAT3 were immunoprecipitated following denaturing cell lysis to remove any STAT-associated proteins and inactivate deubiquitylating enzymes. Immunoblotting of STAT3 immunoprecipitates with anti-ubiquitin antibody revealed that HUVEC treatment with sIL-6Rα/IL-6 only resulted in the accumulation of a smear of ubiquitylated STAT3 in A_{2A}AR-overexpressing cells. Thus, the accumulation of ubiquitin-conjugated STATs in HUVECs occurred under conditions that also promoted their degradation.

(4) To determine the relationship between STAT ubiquitylation and Tyr phosphorylation in A_{2A}AR-overexpressing cells, Flag-tagged WT and Tyr705→Phe mutated STAT3 were co-expressed in HUVECs with the A_{2A}AR and immunoprecipitated with anti-Flag antibody-conjugated Sepharose beads following

denaturing cell lysis after cytokine treatment. These experiments demonstrated that under conditions in which recombinant WT STAT3 is polyubiquitylated similarly to the endogenous protein in response to sIL-6Rα/IL-6, no ubiquitylation of the Tyr705→Phe mutant could be detected.

(5) To date there is only one mammalian E3 ligase known for mediating STAT degradation which is SLIM protein. However, our results suggest the involvement of another E3 ubiquitin ligase in HUVECs, since we have been unable to detect SLIM message or protein in HUVECs under conditions in which STAT degradation occurs. The PCR technique has determined by serial dilution of SLIM/Mystique plasmid to approximately a few thousand (assuming that 1μg of SLIM plasmid pcDNAA3/HA-SLIM/Mystique contains $1.5x10^{11}$ copies of target DNA). For the immunoblotting experiment the Ab was able to detect the positive control (which was lysates from HEK293 cells transfected with SLIM/Mystique cDNA), but the endogenous SLIM/Mystique which may be induced in A_{2A}AR overexpressing cells was below the detection limit of the Ab.

(6) The results showed that there is a siginficant difference in SOCS3 expression between GFP and A_{2A}AR overexpressing cells upon cytokines stimulation at 2 and 3 hrs (Figure 4.12). However, this does not rule out the physiological function of SOCS3 or other SOCS family members in potentially mediating the down-regulation of STAT1 and STAT3.

The limitations of approaches in Chapter 4 are the same as in Chapter 3 for the use of HUVECs and overexpression of A_{2A}AR (pages 102-103).

Chapter 5

Effect of A_{2A}AR Expression on STAT3 Regulated Genes

5.1 Introduction

VEGFR-2/Flk-1/KDR is exclusively expressed in endothelial cells and appears to play a pivotal role in endothelial cell differentiation and vasculogenesis (Millauer *et al.*; 1993; Quinn *et al.*; 1993). Many studies have provided evidence for the role of VEGFR-2/Flk-1/KDR in tumour vascularisation, growth, and metastasis. For example, the manipulation of the cloned receptor to create a "dominant negative" mutation is one experimental technique that has helped establish the relevance of Flk-1 to tumour angiogenesis. The biological relevance of the VEGFR-2/Flk-1/KDR receptor/ligand system for tumour-associated angiogenesis in *vivo* has been demonstrated using a retrovirus encoding a dominant-negative Tyr705→Phe mutant of the VEGFR-2 (inhibit kinases), which prevented the growth of a transplanted glioblastoma (Millauer *et al.*; 1994). Recently, using an anti-sense oligonucleotids directed against Flk-1 and Flt-1, it has been shown that VEGF stimulates, endothelial cell proliferation, migration, and platelet-activating factor synthesis via VEGFR-2. Inhibition of Flt-1 expression failed to affect VEGFs ability to modulate these activities (Bernatchez *et al.*; 1999). These studies have validated targeting of the VEGFR-2 signalling pathway for the development of antiangiogenic agents.

The JAK/STAT and PI-3΄-K/Akt are two parallel pathways responsible for mediating many downstream functions of many receptor and nonreceptor tyrosine kinases, including EGFR, Her-2 and c-Src (Liu *et al.*; 1998; Laughner *et al.*; 2001; Yu and Jove; 2004). gp130, which is frequently activated in a wide range of cancers (Hideshima *et al.*; 2004), also signals through both JAK/STAT and PI-3΄-K/Akt pathways (Falcone *et al.*; 1999; Hideshima *et al.*; 2001). Over-expression and/or persistent activation of EGFR/Her-2, Src and IL-6R are known to promote tumour growth/survival and to induce VEGF expression and angiogenesis (Laughner *et al.*; 2001; Semenza; 2003; Yu and Jove; 2004). As IL-6 activates PI-3΄-K/Akt via SHP2 activation by gp130 this leads to activation of downstream kinases such as Akt (Hideshima et al., 2001). Interestingly, it has been shown that blocking STAT3, but

not PI-3́-K activity, inhibits VEGF expression in tumour cells with constitutive IL-6 signalling (Wei *et al.*; 2003), suggesting that STAT3 continues to activate VEGF expression in the absence of PI3K/Akt signalling.

EC dysfunction is characterised by decreased bioavailability of NO, caused in part by increased oxidant stress and by decreasing NO synthesis (Cai and Harrison; 2000; Fulton *et al.*; 2001). In the vasculature, NO is normally synthesised by the endothelial isoform of eNOS, where it plays a protective role by inhibiting leukocyte trafficking and by decreasing platelet adhesion and aggregation (Fleming and Busse; 1999; Stuehr; 1999; Dudzinski *et al.*; 2006). NO production by endothelial cells is regulated by changes in eNOS enzyme activity and gene expression. The expression of eNOS can be regulated by biophysical stimuli (such as shear stress or hypoxia), growth factors (such as TGF-β1, FGF, VEGF, or PDGF), hormones (such as estrogens, insulin, angiotensin II, or endothelin 1) (Li *et al.*; 2002), or NO itself (Dudzinski *et al.*; 2006) and IL-6 (Saura *et al.*; 2006).

The results in Chapters 3 and 4 suggest that A_{2A}AR overexpression primes JAK-phosphorylated STATs for polyubiquitylation and subsequent degradation by the proteasome. For the down-regulation of STAT proteins observed in A_{2A}AR-overexpressing cells to be considered functionally significant, the ability of cytokines to promote the accumulation of STAT-regulated target gene products should be compromised. In the course of our studies, we identified the vascular endothelial growth factor (VEGF) receptor VEGFR-2 and eNOS as proteins in HUVECs whose levels are controlled by STAT3. Since multiple studies have shown that elevated levels of STAT3 phosphorylation are associated with increased expression of potential downstream targets of STAT3, such as VEGF (Turkson; 2004) and down regulation of eNOS in ECs (Saura *et al.*; 2006). Inhibition of constitutively active STAT3 signalling pathways may inhibit inflammation and tumour cell growth *in vitro* and *in vivo* as well as provide a novel means for therapeutic intervention in

human diseases. In this Chapter we investigate the effect of A_{2A}AR overexpression on the physiologically relevant genes regulated by STAT3 in HUVECs.

5.2 Results

The STATs function as downstream effectors of cytokine and growth factor receptor signalling. Compared with normal cells and tissues, constitutively activated STATs have been detected in a wide variety of human cancer cell lines and inflamed tissue. STATs are activated by tyrosine phosphorylation, which is normally a transient and tightly regulated process (Heinrich *et al.*; 2003). The down-regulation of STAT proteins observed in A_{2A}AR-overexpressing cells would be predicted to modify the ability of cytokines to promote STAT-induced target gene expression. VEGFR-2 is a protein in ECs whose levels are positively controlled by STAT3. Incubation of HUVECs with sIL-6Rα/IL-6 for 4 hr increased VEGFR-2 (Figure 5.1). The cytokine dependence of STAT degradation in A_{2A}AR-overexpressing cells (see Chapter 3) raised the possibility that JAK-mediated STAT phosphorylation was a trigger for this event. JAK inhibition experiments demonstrated that 0.1 μM of JAK inhibitor 1 was sufficient to inhibit Tyr-phosphorylation of JAKs and STATs upon sIL-6Rα/IL-6 stimulation. In this Chapter I wanted to test the effect of JAK inhibition on VEGFR-2 expression when HUVECs were stimulated with sIL-6Rα/IL-6. My results show that, expression of VEGFR-2 upon sIL-6Rα/IL-6 stimulation was blocked by JAK inhibitor 1 pre-treatment (Figure 5.2). Furthermore, in Chapter 4 we demonstrated that JAK-mediated phosphorylation of STATs was the critical step triggering down-regulation when HUVECs were co-infected with AVs encoding the A_{2A}AR and either Flag epitope-tagged WT or Tyr705→Phe mutated STAT3, since mutation of Tyr705 renders STAT3 resistant to phosphorylation by JAK (Kaptein *et al.*; 1996). In this Chapter expression of VEGFR-2 was abolished by overexpression of a dominant-negative Tyr705→Phe STAT3 mutant and produced by overexpression of WT STAT3 (Figure 5.3). The results show that A_{2A}AR overexpression has two effects on VEGFR-2 expression. The first effect was incubation of A_{2A}AR-overexpressing cells

with sIL-6Rα/IL-6 for 4 hr triggered a 91±6% down regulation of VEGFR-2 compared to levels in untreated controls (Figure 5.4). The second effect was that over-expression of the A_{2A}AR alone increased VEGFR-2 expression (Figur 5.4), although this phenomenon appears to be STAT-independent since receptor expression alone produces no detectable changes in STAT phosphorylation (see Chapter 3). The data are consistent with the hypothesis that A_{2A}AR overexpression can prime cytokine-activated STATs for degradation by the proteasome. Also, A_{2A}AR-overexpression was able to down regulate VEGFR-2 expression. This may occur via inhibition of STAT3 regulation of VEGF expression.

NO produced by endothelial cells plays a crucial role for regulation of many biological functions such as vasodilatation, host defense, tissue respiration, and substrate utilisation (Moncada *et al.*; 1991; Trochu *et al.*; 2000). Many studies have show that IL-6 can decrease eNOS expression in ECs. It has been shown that IL-6 treatment of human aortic endothelial cells (HAEC) decreases steady-State levels of human eNOS mRNA and protein and this decrease in eNOS expression is caused in part by IL-6 inhibition of transactivation of the human eNOS promoter (Saura *et al.*; 2006). cAMP signal transduction is a novel pivotal mechanism for regulation of endothelial NO production and may play a crucial role in the control of cardiovascular function. For example, many studies have shown that heart failure is associated with a depressed systemic and cardiac endothelial NO production, and defective endothelial NO formation has been recognised as an important mechanism contributing to the progressive deterioration of this disease (Katz *et al.*; 1993; Mohri *et al.*; 1997; Zhang *et al.*; 1999b). To investigate if over-expression of the A_{2A}AR could regulate eNOS expression, several experimental approaches were used. The data shown in Figure 5.5 shows that levels of eNOS were reduced by transient overexpression of WT STAT3. In contrast, eNOS levels were up-regulated by over-expression of a dominant-negative Tyr705→Phe mutated STAT3. From Figure 5.6 it can be seen that there is no significant change in eNOS expression when the cells are incubated with sIL-6Rα/IL-6. However incubation of A_{2A}AR-overexpressing cells

with sIL-6Rα/IL-6 for 2hr marginally up-regulates eNOS expression compared to controls (Figure 5.7). These results suggest that STAT3 negatively regulates eNOS expression upon sIL-6Rα/IL-6 in A_{2A}AR-overexpressing cells.

Figure 5.1 Effect of sIL-6Rα/IL-6 on VEGFR2 expression

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were serum starving for 4 hrs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodie. Quantitative analysis of VEGFR2 levels from three experiments is presented (***P <0.001 versus the response observed in control cells). Basal set at 100.

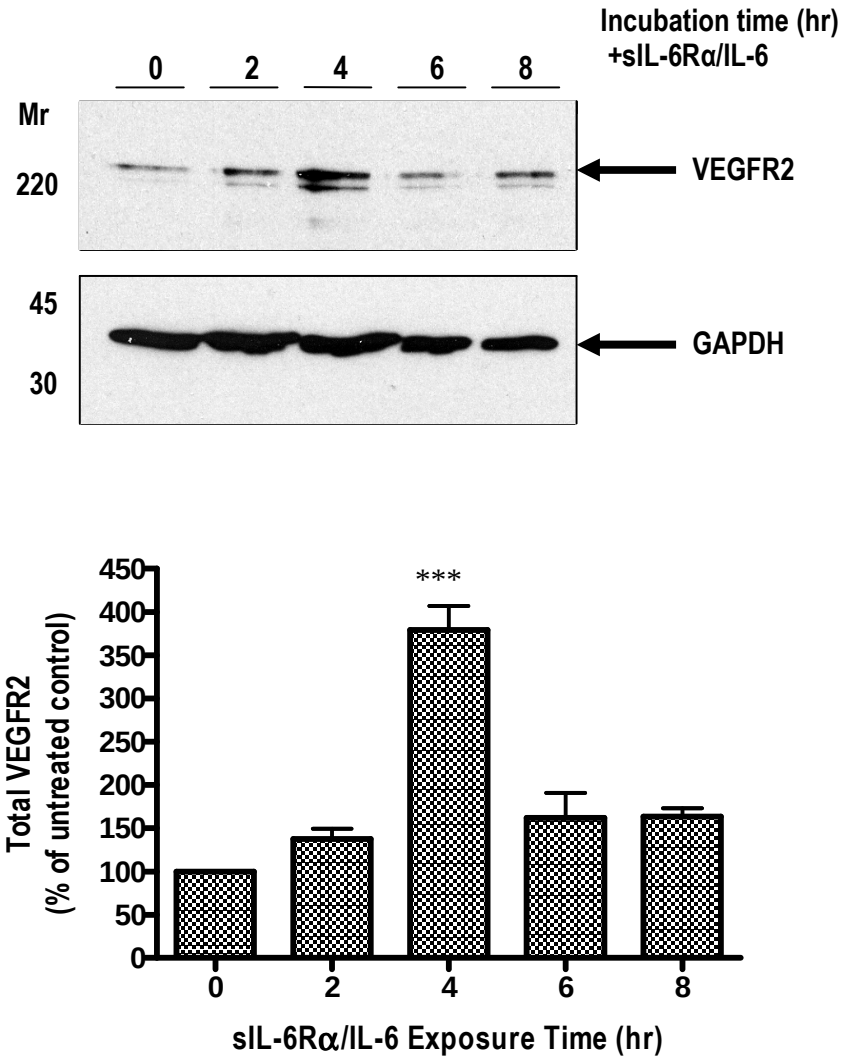


Figure 5.2 Effect of JAK inhibition 1 on sIL-6Rα/IL-6-induced VEGFR2 expression in HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were then serum starving for 4 hrs prior to pre-incubation with or without 0.1 uM JAK inhibitor I for 30 min then treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 30 min as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-VEGFR2 antibody. (**P <0.005 and ***P <0.001 versus the response observed in control cells). Basal set at 100.

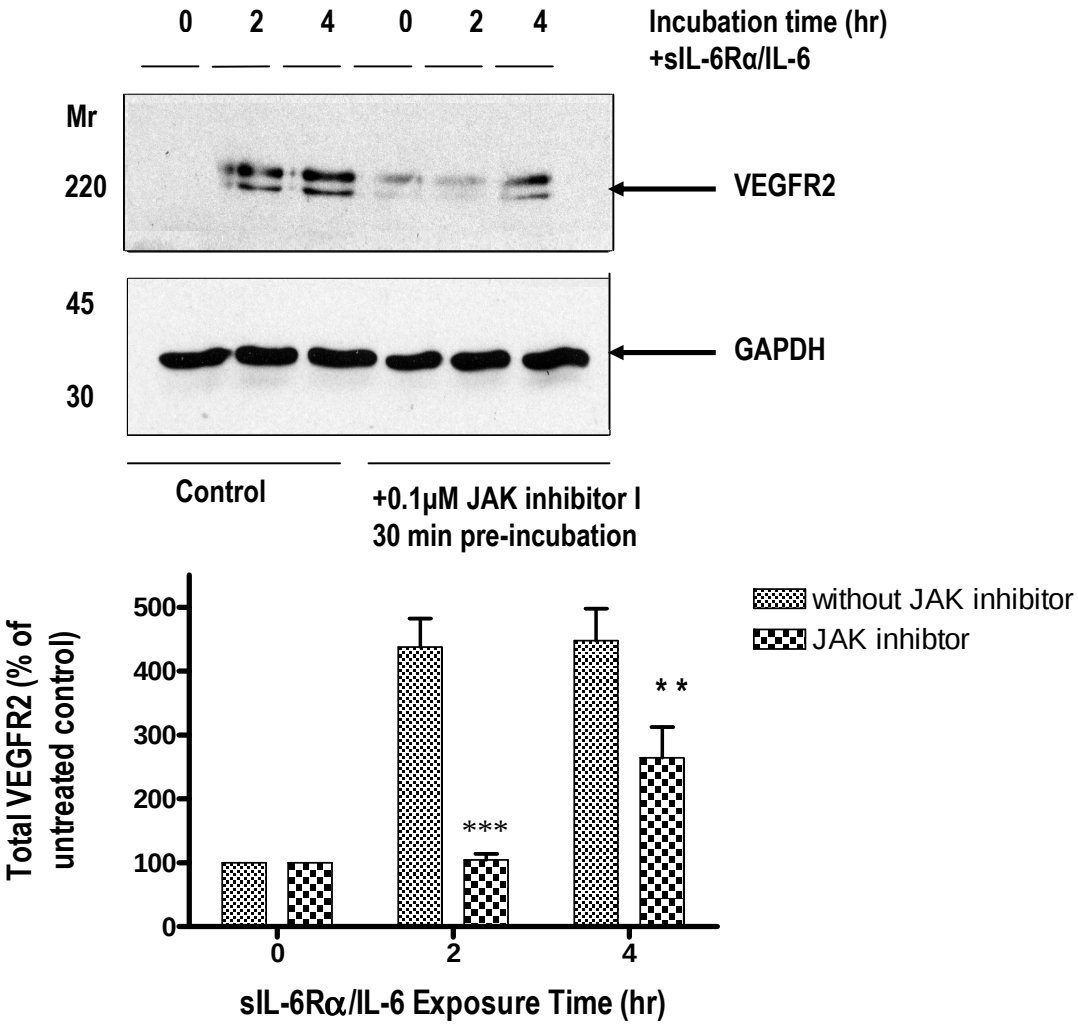
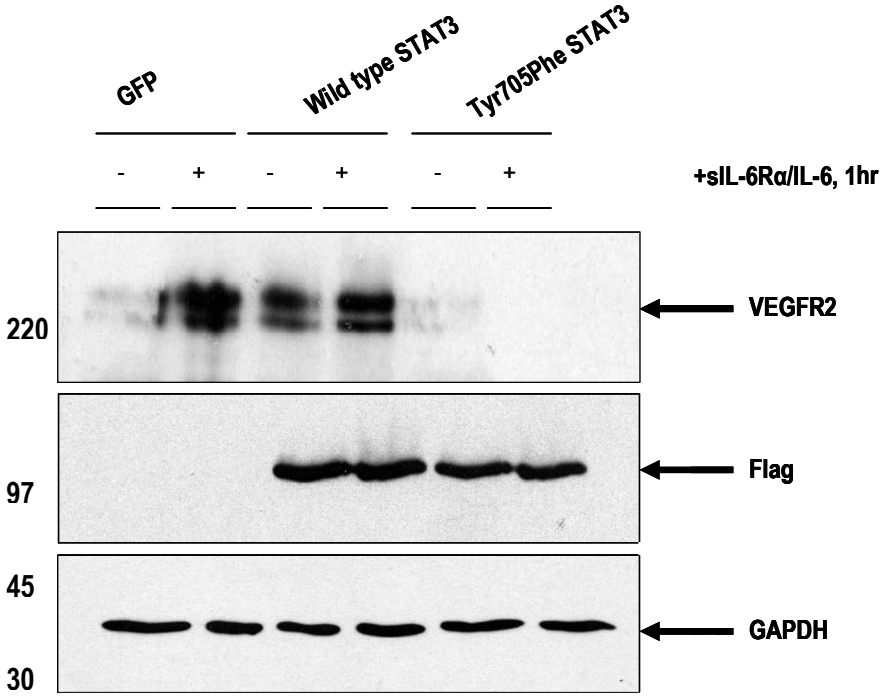


Figure 5.3 Effect of WT and Tyr705→Phe mutated STAT3 expression on VEGFR2 expression in HUVECs

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were co-infected with AV. Flag-WT STAT3 or AV.Flag-Tyr705→Phe mutated STAT3 prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 1 hr as indicated. Soluble cell exracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies (***P <0.001 versus the response observed in mutant STAT3). Basal set at 100.


GFP
Wild type STAT3
Tyr705Phe STAT3
- + - + - +
+sIL-6Rα/IL-6, 1hr
220
97
45
30
VEGFR2
Flag
GAPDH

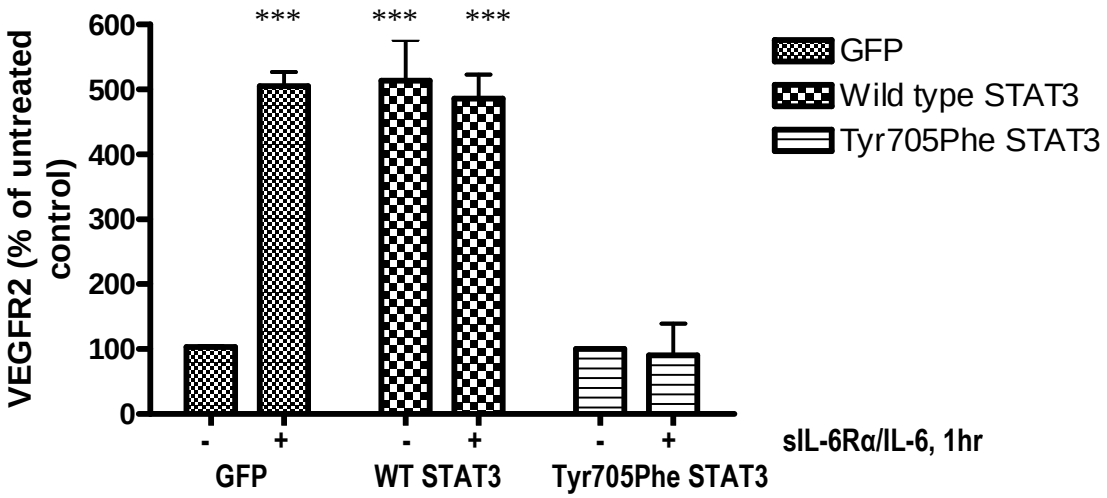

VEGFR2 (% of untreated control)
600
500
400
300
200
100
0
***
***
***
GFP
Wild type STAT3
Tyr705Phe STAT3
- + - + - +
sIL-6Rα/IL-6, 1hr
GFP
WT STAT3
Tyr705Phe STAT3

Figure 5.4 Effect of A_{2A}AR gene transfer on VEGFR2 expression in response to sIL-6Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to serum starving for 4hr and treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of VEGFR2 down-regulation in A_{2A}AR-expressing cells from three experiments is presented (***P <0.001 versus the response observed in AV.GFP infected cells) Basal set at 2 hrs.

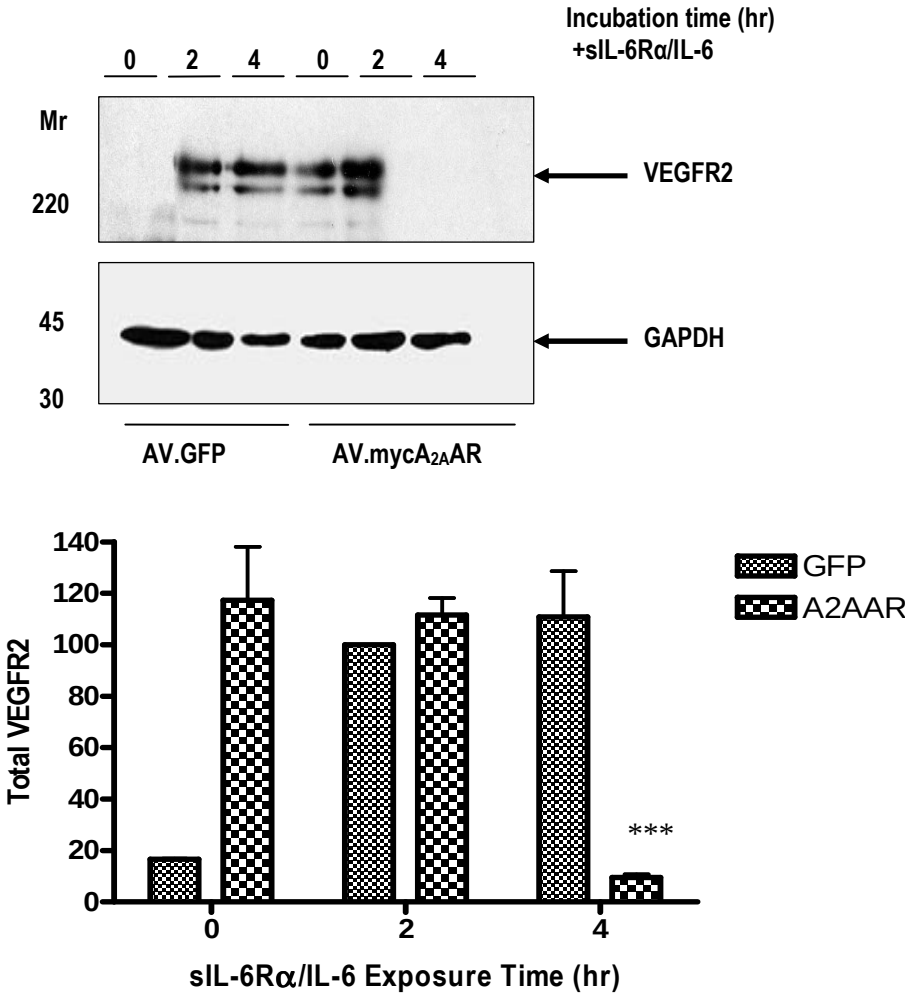


Figure 5.5 Effect of WT and Tyr705→Phe mutated STAT3 expression on eNOS expression

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were co-infected with AV. Flag-WT STAT3 or AV.Flag-Tyr705→Phe mutated STAT3 prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for 1 hr as indicated. Soluble cell exracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies (***P <0.001 and *P <0.05 versus the response observed in WT STAT3). Basal set at 100.

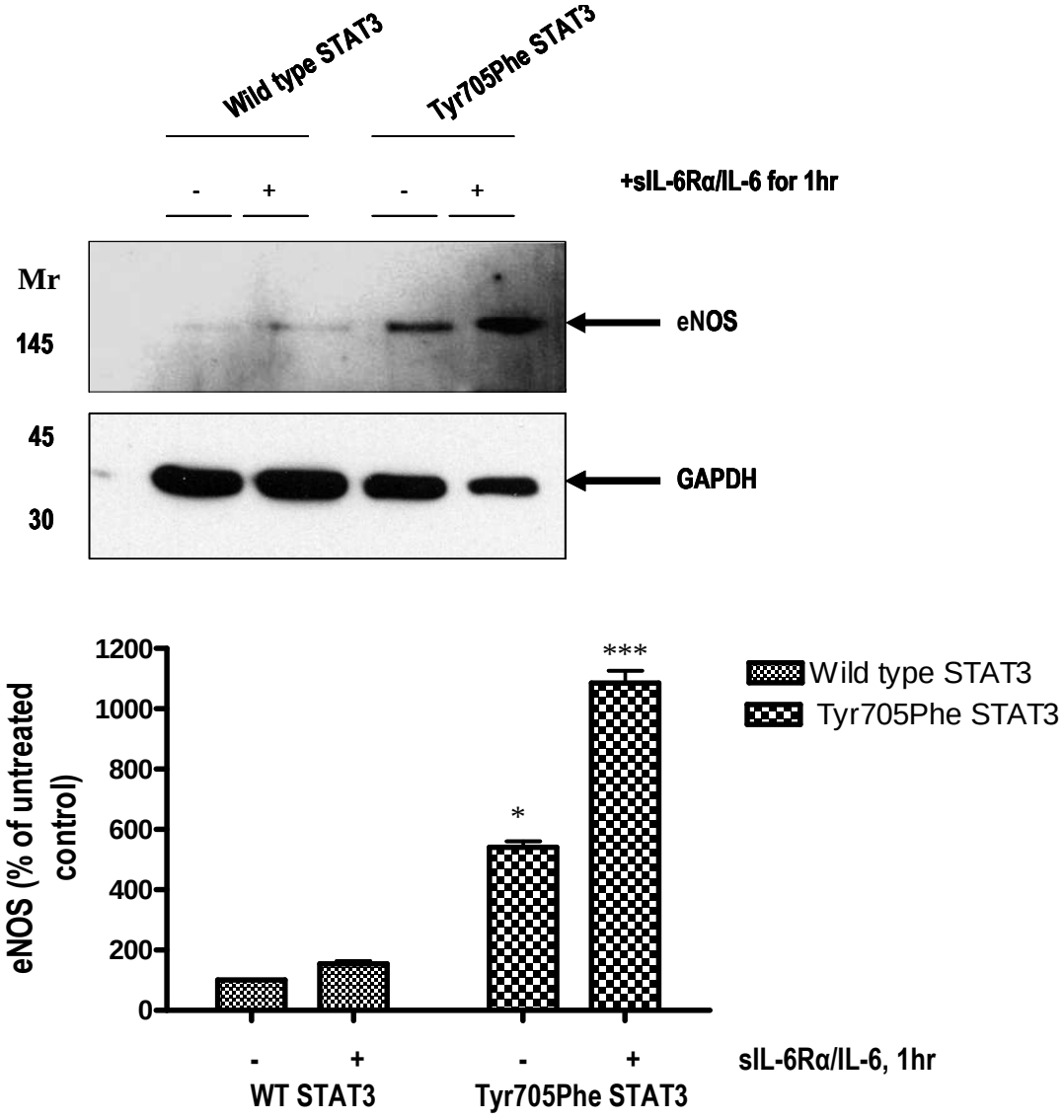


Figure 5.6 Effect of sIL-6Rα/IL-6 on eNOS expression

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were serum starving for 4 hrs prior to treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Basal set at 100.

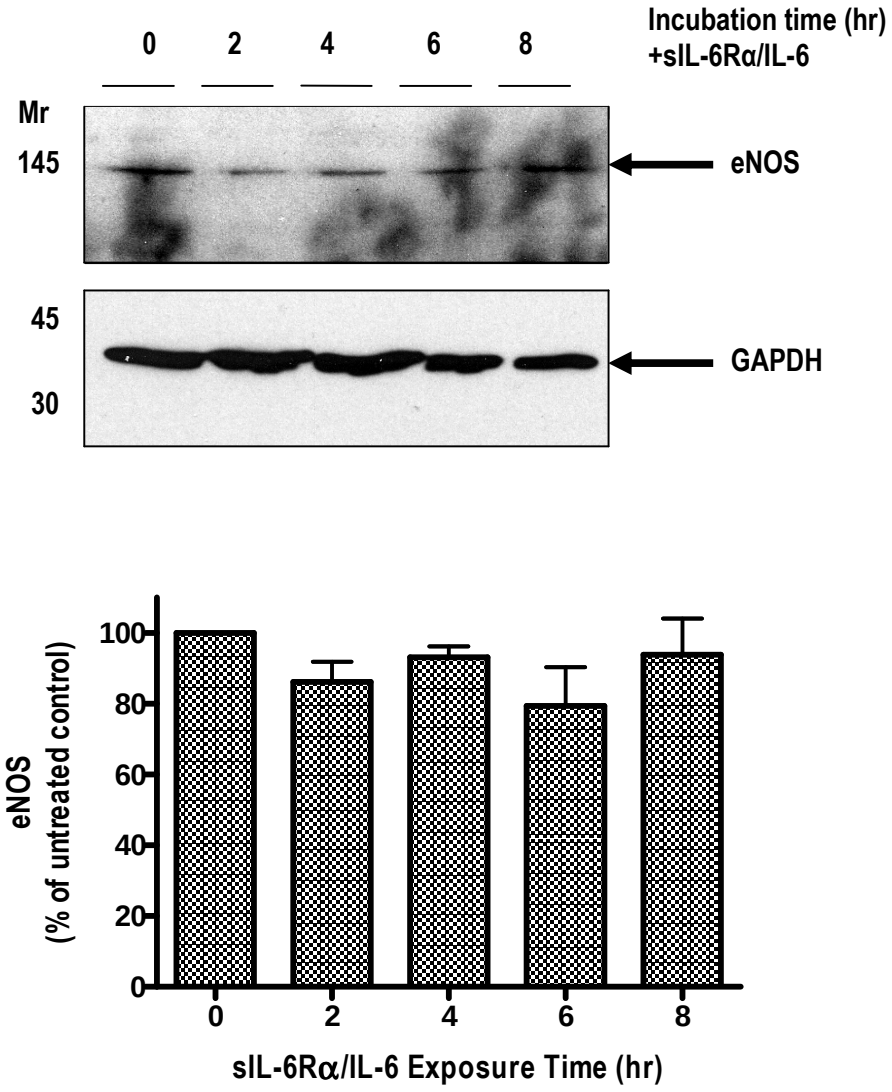
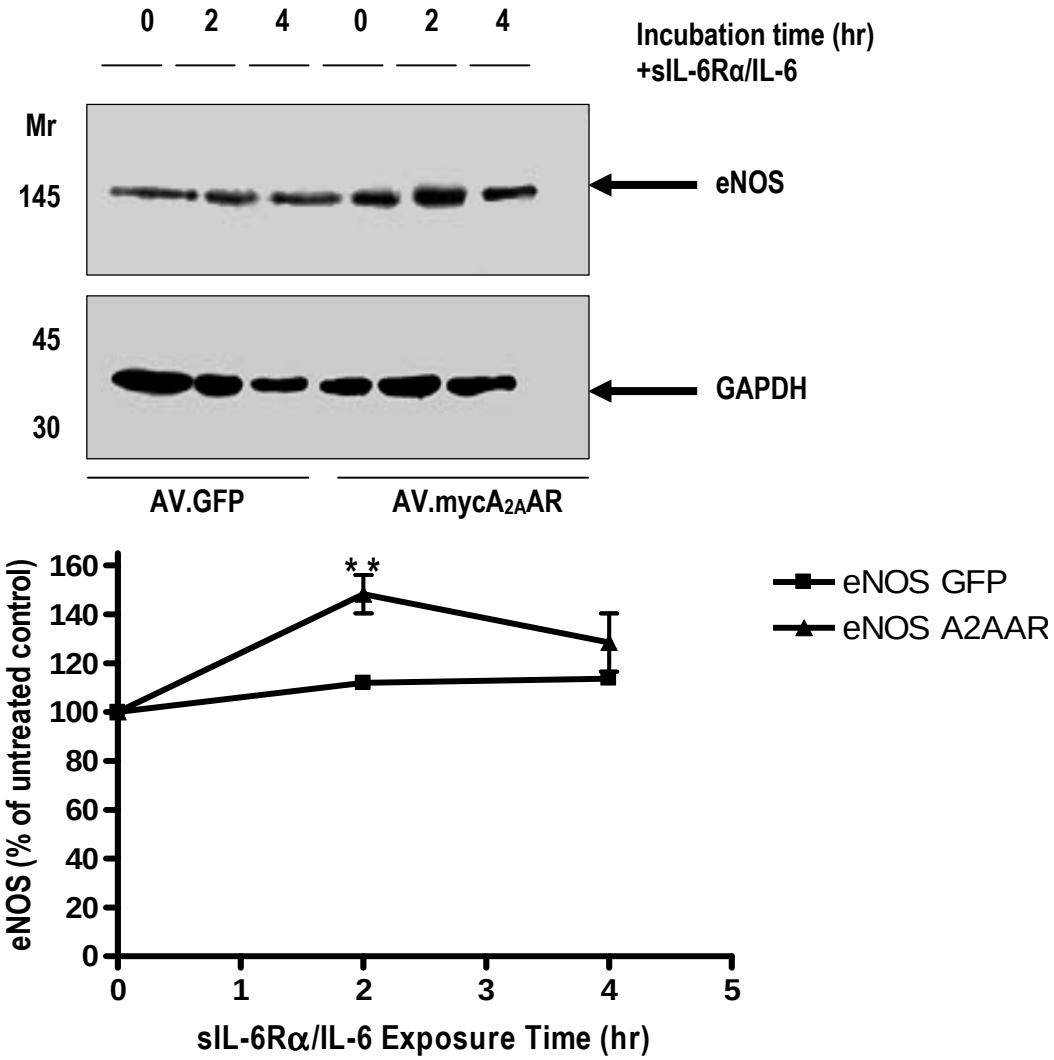


Figure 5.7 Effect of A_{2A}AR gene transfer on eNOS expression in response to sIL-6Rα/IL-6

$1x10^5$ HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were infected with the indicated AVs prior to serum starving for 4hr and treatment with or without 25ng/ml sIL-6 Rα/5ng/ml IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. (**P <0.005 versus the response observed in AV.GFP infected cells). Basal set at 100.



5.3 Discussion

VEGF is up-regulated by multiple stimuli, including hypoxia (Levy *et al.*; 1995b; Levy *et al.*; 1995a), growth factors (Nauck *et al.*; 1997), and cytokines (Li *et al.*; 1995; Ryuto *et al.*; 1996). Conversely, few agents have been shown to reduce VEGF expression as described presently for A_{2A}AR agonists. Glucocorticoids inhibit the up-regulation of VEGF induced by stimuli such as serum, platelet-derived growth factor, phorbol esters, and platelet-activating factor in different cell types (Finkenzeller *et al.*; 1995; Heiss *et al.*; 1996; Nauck *et al.*; 1997).

VEGFR2 is one of the major regulators of vasculogenesis and angiogenesis. The detailed analysis of the role of VEGFR2-mediated signal transduction suggested that the inhibition of VEGFR pathway would provide a powerful antiangiogenic signal that could be highly useful in inhibiting pathogenic angiogenesis To date, several small molecule VEGFR2 kinase (KDR) inhibitors have been tested in animal cancer models and resultant changes in tumor vasculature have been described. Strong anti-angiogenic responses are induced by SU5416 (a potent inhibitor of VEGFR protein kinases) (Vajkoczy *et al.*; 1999; Laird *et al.*; 2000). While the mechanism by which A_{2A}AR activation results in VEGFR2 down-regulation has begun to be explored, our results show that A_{2A}AR overexpression down-regulated VEGFR-2 under the same conditions that promoted STAT degradation after cytokine stimulation (see Chapter 3). In A_{2A}AR-overexpressing cells, the ability of the A_{2A}AR to stimulate down regulation of VEGFR-2 after 4hr of sIL-6Rα/IL-6 stimulation was significant compared with control cells. The expression of VEGFR-2 upon sIL-6Rα/IL-6 was inhibited by JAK inhibitor 1, abolished by over-expression of Tyr705→Phe mutated STAT3 and induced by overexpression of WT STAT3. Several studies have reported that VEGF-induced proliferation is mediated by the interaction of VEGF with VEGFR2 in both breast cancer cells and in endothelial cells (Mercurio *et al.*; 2004; Liang and Hyder; 2005). However, the role of A_{2A}AR in VEGFR2 regulation may be complex. In addition VEGFR2 is exclusively expressed in endothelial cells and

appears to play a pivotal role in endothelial cell differentiation and vasculogenesis (Millauer *et al.*; 1993; Quinn *et al.*; 1993). It has also been demonstrated that VEGFR-2 signals through STAT3 (Bartoli *et al.*; 2003). Blocking STAT3 in endothelial cells inhibit their migration and vessel formation (Yahata *et al.*; 2003). Interestingly, a relationship between tumour STAT3 activity and STAT3 signalling has been recently described (Wang *et al.*; 2004). Our results in Chapter 3 demonstrated that the human A_{2A}AR overexpression was able to confer an ability of sIL-6Rα/IL-6 to trigger a time-dependent reduction in the levels of STAT proteins that was due entirely to proteasomal degradation. In terms of functional consequences, the results of this Chapter show that the degradation was associated with an attenuation of sIL-6Rα/IL-6-stimulated STAT3-dependent up-regulation of VEGFR2. Blocking STAT3 signalling in either tumour or dendritic cells abrogates tumour-induced inhibition of dendritic cell maturation (Wang *et al.*; 2004). Thus, STAT3 plays a central role in propagating oncogenic signals from tumour cells to effector cells involved in tumour angiogenesis and immune evasion. It is a well established fact that blocking STAT3 signalling in tumour cells inhibits tumour cell proliferation and induces apoptosis (Darnell; 2002; Yu and Jove; 2004). In diverse human cancers displaying dependence on persistently activated STAT3 for survival/proliferation (Darnell; 2002; Yu and Jove; 2004), targeting STAT3 is expected to evoke potent anti-tumour effects through direct tumour cell death, anti-tumour immune responses and anti-angiogenesis. PC12 cells, which express A_{2A} and A_{2B}ARs (Arslan *et al.*; 1999), have been employed to study AR signal transduction and physiological activity. It has been shown previously that activation of the A_{2A}AR in PC12 cells results in a substantial reduction of VEGF, which is observed at both the mRNA and protein levels. Furthermore, this down-regulation of VEGF mRNA occurs because of an inhibition of VEGF gene transcription (Olah and Roudabush; 2000). The nonselective AR agonist, 5-(*N*-ethylcarboxamido) adenosine, was also reported to down-regulate VEGF expression in PC12 cells (Kobayashi and Millhorn; 1999). Other cell types have been shown to respond to AR agonists with either increases or decreases in VEGF expression (Grant *et al.*; 1999; Wakai *et al.*; 2001;

Feoktistov *et al.*; 2002). This differential regulation may exist because of the subtype specificity of various AR ligands, and because of cell-specific variations in the signal transduction cascade to which a distinct AR subtype may be linked. Stimulation of the A_{2A}AR in PC12 cells substantially reduces VEGF mRNA expression and VEGF protein secretion. Regulation of VEGF secretion by the A_{2A}AR, or perhaps other G protein-coupled receptors, on selected targets may represent a means to positively or negatively regulate angiogenesis for therapeutic benefit. In this study A_{2A}AR was able to down-regulate the VEGFR-2 under the same conditions promoted STAT degradation after cytokines stimulation.

It has been demonstrated that adenosine enhances IL-1-induced NO production through activation of A_{2A}AR (Ikeda *et al.*; 1997a; Ikeda *et al.*; 1997b; Dubey *et al.*; 1998). In addition, activation of the A_{2A}AR can increase NO production in porcine coronary endothelial cells (Olanrewaju and Mustafa; 2000) and in HUVECs (Wyatt *et al.*; 2002). In this study, we have demonstrated that A_{2A}AR overexpression increases eNOS induction in A_{2A}AR overexpressing cells. This increase in eNOS induction is consistent with our results in Chapter 3 which show that overexpression of the A_{2A}AR triggers the down-regulation of STAT after treatment with sIL-6Rα/IL-6 for 3hr. In addition our results show that Tyr705→Phe-mutated STAT3 enhances eNOS expression, while overexpression of WT STAT3 reduces eNOS expression. Different studies have found that inflammatory mediators decrease eNOS expression (Marsden *et al.*; 1992; Weiss *et al.*; 1994; Tai *et al.*; 2004). Furthermore, TNF-α can decrease eNOS expression by inhibiting eNOS promoter transactivation and also by destabilising eNOS mRNA (Yoshizumi *et al.*; 1993; Alonso *et al.*; 1997; Searles; 2006). CRP, an effector of the acute phase response, decreases eNOS expression by destabilising its mRNA (Venugopal *et al.*; 2002; Verma *et al.*; 2002). IL-6, a major trigger of the APR, has been shown to decrease eNOS expression. Recent studies point to IL-6 as a marker of cardiovascular disease as well as systemic inflammation (Tracy *et al.*; 1997; Libby; 2001; Ridker *et al.*; 2001). IL-6 plasma levels are elevated in myocardial infarction, unstable angina, and atherosclerosis. Chronic inflammation

may trigger synthesis of IL-6, which activates STAT3. STAT3 in turn drives transcription of acute phase response reactants, such as CRP that contribute to the development and progression of atherosclerosis (Verma *et al.*; 2005; Paffen and DeMaat; 2006). Accumulating evidence demonstrates that the A_{2A}AR increases the production of NO by human and porcine arterial endothelial cells, which in turn leads to vasodilation (Lin *et al.*; 2007).

Our results show that incubation of A_{2A}AR-overexpressing cells with sIL-6Rα/IL-6 for 2 hr up-regulates eNOS expression compared with control cells. This result is consistent with finding that adenosine-induced NO production is blocked by the potent eNOS inhibitor l-NIO (Jiang *et al.*; 2002; Benamar *et al.*; 2003) but not by the selective iNOS inhibitor l-NIL (Connor *et al.*; 1995), suggesting eNOS but not iNOS is responsible for the effect of adenosine on NO production. The importance of eNOS in NO production by adenosine is further supported by the observation that adenosine enhances phosphorylation of eNOS at its activation sate (Ser 1177) (Albrecht *et al.*; 2003; Zhang and Hintze; 2006). Moreover, adenosine significantly increased eNOS, but not iNOS, activity (Albrecht *et al.*; 2003). It has been suggested that a large amount of NO produced by iNOS is toxic, whereas eNOS is a protective enzyme (Albrecht *et al.*; 2003). Therefore, it is highly likely that adenosine-induced NO generation through eNOS can result in cardioprotection, which may explain the cardioprotective effect of adenosine. Multiple lines of evidence have suggested that the A_{2A}AR is critical for adenosine-mediated protection against ischaemia-reperfusion injury. A_{2A}AR-mediated inhibition of tissue ischaemia-reperfusion injury has been documented in various organ systems, including liver, lung, kidney, and heart (Okusa *et al.*; 1999; Harada *et al.*; 2000). However, the precise mechanisms responsible for A_{2A}AR-mediated tissue protection remain unknown. The A_{2A}AR is widely distributed and mediates a variety of physiological responses in mammals. A_{2A}ARs couple to Gs proteins and activate adenylyl cyclase, leading to an increase in cellular cAMP levels (Dobson and Fenton; 1997; Sullivan *et al.*; 2001). The mechanisms of protection mediated by A_{2A}AR activation may include inhibition of

leukocyte-mediated inflammatory response, chemokine production and vasodilation (Shryock *et al.*; 1998; Lew and Kao; 1999). A recent study has also shown that cAMP induces eNOS activation and increases endothelial NO production from isolated canine and porcine coronary microvessels (Kudej *et al.*; 2000; Zhang *et al.*; 2002b). In cultured endothelial cells, both PKA and PKB have been reported to have effects on eNOS phosphorylation and activation (Dimmeler *et al.*; 1999; Fulton *et al.*; 1999; Michell *et al.*; 2001; Boo *et al.*; 2002). These observations may suggest that expression of A_{2A}ARs elevate cAMP which induce eNOS expression as our results would suggest A_{2A}AR-expressing cells up-regulates eNOS expression (Figure 5.4). Thus the data suggest there are two possible mechanisms; the first one is cAMP via ERK directly up-regulate eNOS. The second mechanism is cAMP up-regulates eNOS expression via down-regulation of STAT3. In summary the results suggest that STAT3 regulates eNOS expression upon sIL-6Rα/IL-6, since A_{2A}AR overexpression which primes cytokine-activated STATs for degradation by the proteasome, was sufficient to up-regulate eNOS expression.

Data in Chapter 5 reveals several important aspects. These include:

(1) Incubation of HUVECs with sIL-6Rα/IL-6 for 4 hr increased VEGFR-2 protein levels.

(2) Expression of VEGFR-2 upon sIL-6Rα/IL-6 stimulation was blocked by JAK inhibitor 1 pre-treatment.

(3) Expression of VEGFR-2 was abolished by over-expression of a dominant-negative Tyr705→Phe STAT3 mutant and induced by overexpression of WT STAT3.

(4) The results show that A_{2A}AR overexpression has two effects on VEGFR-2 expression. The first effect was incubation of A_{2A}AR overexpressing cells with sIL-6Rα/IL-6 for 4 hr triggered a 91±6% down regulation of VEGFR-2 compared to

levels in untreated controls. The second effect was that over-expression of the A_{2A}AR alone increased VEGFR-2 expression.

(5) The result shows that levels of eNOS were reduced by transient overexpression of WT STAT3. In contrast, eNOS levels were up-regulated by overexpression of a dominant-negative Tyr705→Phe mutated STAT3.

(6) There is no significant change in eNOS expression when the cells are incubated with sIL-6Rα/IL-6.

(7) Incubation of A_{2A}AR-overexpressing cells with sIL-6Rα/IL-6 for 2 hr marginally up-regulates eNOS expression compared to controls.

In the course of my studies, I have identified the vascular endothelial growth factor (VEGF) receptor VEGFR2 and eNOS as proteins in ECs whose levels are controlled by STAT3. The interaction between the eNOS promoter and STAT3 has been studied by Marta Saura *et al.* (2006). They first studied the effects of IL-6 upon the human eNOS 5′-flanking region extending 1600 bp upstream from the transcriptional start site. Then, they transfected HAEC with an eNOS promoter luciferase construct (eNOS-Luc). While Control cells were also co-transfected with plasmid constitutively expressing *Renilla* luciferase. Then, human eNOS promoter transactivation was measured as firefly luciferase activity normalised to *Renilla* luciferase activity. Their data show that STAT3 upon IL-6 activation inhibits eNOS promoter activity in endothelial cells in a dose-dependent manner. While any interaction between STAT3 and the VEGFR2 promoter has yet to be identified, several experiments could be used to address this issue. One approach is chromatin immunoprecipitation (ChIP) analysis which could be applied to assess cytokine-inducible STAT3 recruitment to the VEGFR2 promoter. To perform this experiment, cells have to be stimulated with and without IL-6 and then fixed. Then DNA has to be sheared to fragment sizes of roughly 500 bp. STAT3 is then immunoprecipitated and DNA fragments analysed by

PCR. Primers would be designed to amplify a target sequence of no greater then 200 bp that incorporates any putative STAT3 binding sites in the VEGFR2 promoter. Secondly, reporter gene assays could be employed to confirm IL-6-inducible STAT3 activation of VEGFR2 promoter. This could be done by cloning the VEGFR2 promoter upstream of a luciferase ORF in a mammalian expression vector such as pLuc (Chiou *et al.*; 2000). If cytokine activation of luciferase expression is observed, the STAT sites in the promoter could be mutated and used to compare their ability to induce luciferase following IL-6 stimulation. Additionally, the effect of the dominant-negative Tyr705→Phe STAT3 mutant following IL-6 stimulation could also be assessed as further evidence for a role of STAT3.

The limitations of approaches in this Chapter are the same as in Chapter 3 and 4 for the use of HUVECs and overexpression of A_{2A}AR (pages 102-103).

Chapter 6

Final Discussion

The pro and anti-inflammatory mediators are both meant to be beneficial to the organism. During the initial appearance of pro- and anti-inflammatory mediators in the circulation, the beneficial effects usually outweigh their harmful effects. However, when the balance between these two opposing forces is lost, the mediators become harmful. Sequelae of inappropriate or prolonged inflammation contribute to the pathogenesis of many diseases including atherosclerosis (D'Cruz; 1998; Sands and Palmer; 2005), rheumatoid arthritis (Karouzakis *et al.*; 2006) sepsis, (D'Cruz; 1998; Kinlay *et al.*; 2001; Greaves and Channon; 2002; Gueler *et al.*; 2004), heart disease (Kinlay *et al.*; 2001), and cancer (Howe; 2007). Fundamental to the inflammatory response is the interaction between ECs and leukocytes. This interaction triggers further downstream signalling events leading to cytokine, chemokine and growth factor release, surface expression of adhesion molecules and expression of other pro-inflammatory proteins.

In this investigation, I have demonstrated that the A_{2A}AR overexpression can prime cytokine-activated STATs for polyubiquitylation and subsequent degradation by the proteasome (Figure 3.2-3.9). Similar to the effect observed on suppression of NF-κB in two separate cell systems (Sands *et al.*; 2004), expression of the A_{2A}AR in the absence of any agonist was sufficient to prime STATs for degradation. Pre-incubation with the proteasome inhibitor MG132 was sufficient to abolish the effect of the A_{2A}AR overexpression on priming both STAT1 and STAT3 for down-regulation in response to both cytokines sIL-6Rα/IL-6 and IFNα. Our data reveals several important aspects of A_{2A}AR overexpression on JAK-STAT upon cytokine stimulation. These include: (1) Potentiation of A_{2A}AR function by increasing its expression reduces cytokine receptor activation of the JAK-STAT pathway by priming Tyr-phosphorylated STAT for proteasomal degradation. (2) Over-expression of the A_{2A}AR in HUVECs suppressed the ability of IFNα and a sIL-6Rα/IL-6 trans-signalling complex to promote Tyr-phosphorylation of STATs 1 and 3 by targeting cytokine-activated STATs for proteasomal degradation. (3) Immunoprecipitation and pull-down experiments revealed that endogenous and recombinant WT STAT3 were

ubiquitylated following cytokine treatment of A_{2A}AR overexpressing cells while no detectable ubiquitylation of Tyr705→Phe-mutated STAT3 was observed. Degradation required JAK-mediated phosphorylation of STATs as deduced from three lines of evidence. First, the effect was abolished by a concentration of JAK inhibitor 1 that abolished Tyr-phosphorylation of STAT1 and STAT3. Second, STAT3 but not STAT1 was targeted for degradation following exposure of A_{2A}AR-overexpressing cells to leptin, reflecting the ability of leptin to specifically promote the Tyr-phosphorylation of STAT3. Third, a Tyr705→Phe mutated STAT3 was resistant to both JAK-mediated phosphorylation and cytokine-triggered degradation in A_{2A}AR overexpressing cells.

The multiple signalling employed by the A_{2A}AR to inhibit STAT activity raise an important question. How does the A_{2A}AR affect STAT activity by different mechanism? One possibility is that A_{2A}AR overexpression leads to the activation of multiple distinct signalling molecules, which then affect the STAT pathway by independent mechanism. One key feature of the A_{2A}AR is the long C-terminal tail, which plays a role in regulating the high level of constitutive activity of the receptor (Klinger *et al.*; 2002). In addition it is shown to be involved in the formation of heterodimers with the dopamine D_2 receptors within the rat striatum (Canals *et al.*; 2003), although it is not required for the formation of A_{2A}AR homodimers (Canals *et al.*; 2004). Therefore its precise role in the formation of oligomers is still unclear. In order to understand the molecular events by which STAT degradation occurs, it would an important step to identify which A_{2A}AR-activated signalling pathways are responsible. A_{2A}AR activation has been reported to activate at least two primary signalling cascades in vascular ECs. The first one is the cAMP-adenylyl cyclase (Linden; 2001), causing an elevated levels of intracellular cAMP. cAMP causes the activation of cAMP-dependent protein kinase (PKA) and “exchange protein activated by cAMP” (Epac). Epac functions as a guanine nucleotide exchange factor for the Rap family of small G-proteins (Bos; 2003; Tasken and Aandahl; 2004). The second pathway is extracellular signal-regulated kinase (ERK). cAMP-independent

activation of ERK through the activation of A_{2A}AR (Sexl *et al.*; 1997) is thought to be required for receptor-mediated generation of nitric oxide, which subsequently activates soluble guanylyl cyclase (Wyatt *et al.*; 2002). The resulting accumulation of cyclic GMP (cGMP) activates cGMP-dependent protein kinase. A range of selective inhibitors and activators of relevant intracellular signalling pathways would be needed to be tested for their ability to either block or mimic A_{2A}AR-mediated priming of STAT degradation. For example, a contribution of Epac could be tested by determining the extent to which Epac-selective activator 8-pCPT-2′-O-me-cAMP (Kooistra *et al.*; 2005) can prime STATs for cytokine-triggered degradation, and whether its depletion by siRNA abolished the A_{2A}AR's effect.

To date, only one mammalian E3 Ub ligase (termed "SLIM" or "Mystique") has been demonstrated to target Tyr-phosphorylated STATs for polyubiquitylation (Tanaka *et al.*; 2005). However, the results presented here suggest that there is no involvement of SLIM under conditions in which STAT degradation occurs, suggesting that another E3 is responsible. Identification of this new E3 would constitute a major advance towards designing strategies for manipulating STAT function, and is therefore a key objective. There are several technically distinct strategies that could be used to identify the E3 ligase. First strategy is the STAT3 proteomics. The most straight forward method for identifying the protein responsible would be to purify STAT-associated proteins in cytokine-stimulated A_{2A}AR-overexpressing cells *via* a two-step tandem affinity purification (TAP) procedure (see Chapter 7). Similar approaches have already been used to demonstrate that KLHL12 function as the substrate recognition component of the E3 complex responsible for degradation of Dsh proteins (Angers *et al.*; 2006). The second strategy is using E3 Ub ligase siRNA library screening (see Chapter 7). This approach is particulary suited to HUVECs since our lab and others have been able to achieve almost complete target gene knockdown following siRNA transfection into these cells (Huang *et al.*; 2005; Kooistra *et al.*; 2005).

The A_{2A}AR displays two desirable features that would make it suitable to act as a brake of the inflammatory response. One important feature is the level of constitutive activity displayed by the receptor, an activity which is associated with the C-terminal of the receptor, since its removal can inhibit the level of constitutive activity (Klinger *et al.*; 2002). The constitutive activity of the receptor would allow the receptor to mediate its anti-inflammatory effects even in the absence of agonist as described in this thesis. The constitutive activity of the receptor suggests that regulation of A_{2A}AR expression would be critical in the inhibition of inflammatory responses. Studies in $A_{2A}AR^{-/-}$ cells showed that there was no significant compensatory increase in the expression of any of the other ARs, suggesting that the anti-inflammatory events triggered by A_{2A}AR expression are specific for this receptor (Lukashev *et al.*; 2003). In addition, demonstration of the absence of receptor reserve in murine T-cells suggests that A_{2A}AR expression needs to be tightly regulated (Armstrong *et al.*; 2001). The regulation of A_{2A}AR expression is supported by the reported alterations in expression in response to Th1 cytokines in Ecs is likely to alter cellular responsiveness to inflammatory stimuli (Nguyen *et al.*; 2003). Moreover, in mature plasmacytoid dendritic cells, the A_{2A}AR becomes up-regulated in response to CpG oligodeoxynucleotide activation of TLR-9, leading to a decrease in IL-6, IL-12 and IFNα production (Schnurr *et al.*; 2005). Therefore, there are precedents for the regulation of A_{2A}AR expression by different inflammatory stimuli resulting in the inhibition of inflammatory responses.

A_{2A}AR overexpression leads to the accelerated degradation of Tyr-phpsphorylated STATs. The JAK-STAT signalling cascade plays an important role in the activation of several different inflammatory genes including eNOS and VEGF. Targeting STAT proteins for therapeutic intervention in cancer remains to be fully explored. In addition to the development of tyrosine kinase inhibitors, antisense STAT oligonucleotides, it will be important to consider alternative strategies for targeting of constitutive STAT signalling (Seidel *et al.*; 2000; Turkson and Jove; 2000). Such strategies could potentially include: (1) development of receptor-ligand interaction

antagonists, such as cytokine antagonists and receptor-neutralising antibodies; (2) inhibition of STAT activating tyrosine kinases; (3) activation of STAT-specific phosphatases; (4) targeting of STAT-regulated genes involved in malignant progression; and (5) development of small molecule inhibitors that interfere with STAT dimerisation and/or DNA binding. With regard to the latter, recent progress has been made in design of short peptides that effectively block STAT3 dimerisation and DNA-binding activity both *in vitro* and *in vivo* (Turkson *et al.*; 2001). Importantly, these peptides inhibit cell transformation mediated by activated STAT3 and provide the basis for development of peptidomimetics with drug-like features. For drug development, molecular assays that are designed to specifically measure activated STAT3 DNA-binding or gene-regulatory activities can be applied for drug refinement through structure-activity relationship studies. In the clinical setting, immunohistochemical assays for detection of activated phosphotyrosine-forms of STAT3 and STAT5 can provide convenient molecular markers for monitoring the efficacy of inhibitors of STAT signalling in biopsies from cancer patients. Gene expression profiling by microarray technology is expected to reveal a molecular signature of STAT-regulated genes that may have diagnostic as well as prognostic applications (Turkson and Jove; 2000; Nikitakis *et al.*; 2004).

STAT activation is negatively regulated by SOCS proteins. Our results do not rule out the physiological involvement of SOCS3 in down-regulation of STAT in A_{2A}AR-overexpressing cells. Thus, A_{2A}AR overexpression may up-regulate other SOCS expression such as SOCS1 leading to accelerated STAT1 and STAT3 degradation. SOCS proteins were originally identified as cytokine-inducible SH2-domains-containing proteins (CIS). They are known to inhibit STAT activation by at least two distinct mechanisms that differ between family members: SOCS1 inhibits activation of JAK by directly binding to JAK, while SOCS3 inhibits the action of JAK only when bind to receptors such as gp130 (Section 1.3). Expression of SOCS1 but not SOCS3 promotes the degradation of ubiquitinylated JAK2 leading to the reduction of STAT activation (Shuai and Liu; 2003). Moreover, STAT1 is known to be regulated

by phosphorylation-dependent ubiquitination in response to IFNγ (Kim and Maniatis; 1996). SOCS proteins typically consist of a phosphotyrosine-binding SH2 domain, a C-terminal SOCS-box involved in proteasome recruitment and a pre-SH2 domain that only in the case of SOCS1 and SOCS3 contains a JAK-blocking KIR domain. Association of SOCS proteins with their target substrates is believed to occur solely via their SH2 domain. Given that SOCS can promote the degradation of JAK, it is also possible that SOCS can target the degradation of associated STAT proteins. Therefore A_{2A}AR overexpression may negatively regulate STAT1 and STAT3 protein levels by increasing the levels of SOCS expression. However, results in Chapter 4 suggest there is a significant difference in SOCS3 expression between GFP and A_{2A}AR overexpressing cells upon cytokines stimulation at 2 and 3 hrs (Figure 4.12). However, this does not rule out the physiological function of SOCS3 or other SOCS family members in the down-regulation of STATs. To further identify the precise role for A_{2A}AR-mediated anti-inflammatory effects, immuoblotting using anti-SOCS antibodies or employing $SOCS^{-/-}$ cells could be used to determine the effect of the A_{2A}AR on SOCS expression. In addition, it would be interesting to determine if this represents a common mechanism of action. For example, STAT activation is also stimulated by other cytokines like IL-11, which utilises a common gp130 receptor component. Similar analysis of STAT1 and STAT3 activation and SOCS expression could be used to determine if A_{2A}AR is important in this way. Another important topic for future studies is to define the mechanisms of crosstalk between JAKs and other pathways. For instance, the receptor Notch has been reported to promote STAT3 activation, and the Notch effectors Hes1 and Hes5 have been found to associate directly with JAK2 and STAT3 (Kamakura *et al.*; 2004). Evidence for cooperation between the JAK/STAT and Notch pathways has also been provided by work in *Drosophila* (Josten *et al.*; 2004) and genetic screens in *Drosophila* have identified additional potential modifiers of the JAK/STAT pathway (Bach *et al.*; 2003). JAKs have also been reported to be activated by a variety of structurally diverse receptors beyond the cytokine receptors. Examples include receptor tyrosine kinases, and G-protein-coupled receptors (such as chemokine

receptors). Hyperactivation of STAT1 and STAT3 due to either inactivation of SOCS promoters by methylation (Niwa *et al.*; 2005) or mutationally activated JAK/other STAT-phosphorylating tyrosine kinases has been observed in breast cancer cell lines as well as prostate, ovarian, pancreatic and hepatocellular carcinomas (Verma *et al.*; 2003). Importantly, in many of these cases, blockade of STAT activation triggers apoptosis of the affected cells (Verma *et al.*; 2003; Yu and Jove; 2004). Thus, manipulation of the A_{2A}AR signalling system might provide one strategy with which to arrest tumour growth resulting from inappropriate activation of STATs. In addition, VEGF is up-regulated by multiple stimuli, including hypoxia (Levy *et al.*; 1995b), growth factors (Nauck *et al.*; 1997), and cytokines (Li *et al.*; 1995; Ryuto *et al.*; 1996).

Since Tyr-phosphorylation is clearly the critical step in targeting STATs for degradation in A_{2A}AR overexpressing cells, it is extremely important to clarify whether it functions simply as a classical phosphodegron, or whether the nuclear translocation that occurs as a result of phosphorylation is also important for localising the phosphorylated STAT dimer with the relevant E3 ubiquitin ligase. This may be tested by comparing the extent to which the A_{2A}AR overexpression primes STATs for cytokine-triggered polyubiquitylation and degradation in cytosolic and nuclear fraction (see Chapter 7).

In conclusion**,** the identification in this study of a previously unappreciated mechanism by which a GPCR can negatively control STAT function by targeting tyrosine-phosphorylated STATs for degradation has significant implications for diseases associated with altered regulation of the JAK-STAT pathway in vascular endothelium. It also suggests that potentiation of A_{2A}AR overexpression function might prove a particularly useful strategy with which to down-regulate pro-inflammatory responses in vascular endothelium by virtue of its capacity to inhibit both the JAK-STAT and NF-κB signalling pathways utilised by distinct pro-inflammatory stimuli.

Chapter 7
Future Experimental Approaches

The centeral hypothesis of the future work is that the over-expression of the A_{2A}AR primes STATs for cytokine-triggered polyubiquitylation and protesomal degradation, and that this is achieved by increasing the activity and/or expression of an E3 Ub ligase that specifically targets Tyr-phosphorylated STATs. I also hypothesise that this previously unappreciated process is an important mechanism by which the inflammatory response could be suppressed by this receptor. Processes regulating protein turnover by the UPS are attractive targets for therapeutic intervention but are currently underexploited (Nalepa *et al.*; 2006). Thus, while the machinery controlling STAT degradation in ECs has tremendous potential for the generation of novel anti-inflammatory therapeutics for treatment of CVDs, fully exploiting this opportunity would require a detailed understanding of the molecular mechanisms responsible. To achieve this, future work would be aimed at :

1) Characterising in detail and defining the subcellular localisation of the STATs ubiquitylation and degradation events.
2) Identification the A_{2A}AR-regulated E3 Ub ligase responsible.
3) Identification of Ub-conjugation sites and the nature of any chain linkage on STAT3.
4) Identification of the A_{2A}AR-activated signalling pathway(s) responsible for priming STATs for degradation.

Proposal experimental procedures:

1) Characterisation and localisation of STAT polyubiquitylation and degradation

Having demonstrating that Tyr-phosphorylation is required for cytokine-triggered degradation of STATs following A_{2A}AR overexpression, it is unclear where ubiquitylation and degradation occur. These questions could be answered as follows:-

1.1) Are STATs degraded in the nucleus?

Tyr-phosphorylation is followed by the α-importin-mediated translocation of STAT dimer into the nucleus (Norman and Shiekhattar; 2006). This would be tested by comparing the extent to which the A_{2A}AR overexpression primes STATs for cytokine-triggered polyubiquitylation and degradation in cytosolic and nuclear fractions. Based on these findings, several experimental approaches such as siRNA-mediated knockdown of importin-α (Which play important role in translocation of STATs from cytosol to the nucleus) could be use to test the important of nucleus loclisation in STAT degradation. Moreover, nuclear import of STAT1 has been shown to be mediated by importin-α 5/NPI-1, one of the importin- α family members. Here, we attempt to determine whether the stimulation-induced nuclear import of STAT3 is mediated by importin-α similar to the import of STAT1. The domain structure of importin-α could be divided into an N-terminal region (the importin-β binding domain), a central region (classical NLS binding domain), and a short C-terminal region. It is well known that the N-terminal domain of importin-α is essential for importin-β binding, and that the deletion of this domain causes a loss of import activity. In the case of STAT1, N-terminal deletion mutants of importin-α 5/NPI-1 lack the importin-β binding domain and cannot bind to importin-β, inhibiting the nuclear import of STAT1 (Sekimoto *et al.*; 1997; McBride *et al.*; 2002). Therefore, we could examine the issue of whether the N-terminal deletion mutant of importin-α 5/NPI-1 inhibits the nuclear import of STAT3 and its degradation in A_{2A}AR-overexpressing cells.

During the process of IL-6-mediated transcriptional activation of STAT3, STAT3 proteins translocate into the nucleus and are subsequently exported from the nucleus in a chromosomal region maintenance 1 (CRM1)-dependent manner (Bhattacharya and Schindler; 2003). Use of nuclear export inhibitor leptomycin B (to inhibit STAT nuclear export) and expression of STAT3 mutants in which nuclear localisation or export sequences are disrupted) could be used to determine whether Tyr-

phosphorylation alone is sufficient for STAT ubiquitylation and degradation, or if nuclear localisation is also critical. If degradation occurs in the nucleus, it would be expected that blocking export will cause nuclear accumulation of Ub STAT.

2) Identification of the E3 ligase for STAT ubiquitylation

To date only one E3 ligase termed "SLIM or Mystique" has been demonstrated to target Tyr-phosphorylated STATs for polyubiquitylation (Tanaka *et al.*; 2005). However I have been unable to detect SLIM message or protein under conditions resulting in STAT degradation suggesting that another E3 is responsible. Identification of this new E3 would constitue a major advance towards designing strategies for manipulating STAT function. Two different strategies are proposed:-

A) Tandem affinity purification (TAP) of STAT-associated E3 ligase

The most straightforward strategy for identifying the protein responsible would be to purify STAT-associated protein in cytokine-stimulated A_{2A}AR-overexpressing cells *via* two-step tandem affinity purification (TAP) procedure. Similar approaches have already been used to demonstrate that KLHL12 function as the substrate recognition component of the E3 complex responsible for degredation of Dsh proteins (Angers *et al.*; 2006). To identify the A_{2A}AR-regulated STAT E3, a TAP construct would be generated comprising streptavidin-(Streptag) and Ni^{2+} chelate-(His_6) binding affinity tags placed in tandem at the C-terminus of STAT3 (Tagwerker *et al.*; 2006). The C-terminus is being chosen as others have demonstrated that even the addition of large fluorescent protein tags at this terminus does not compromise STAT3 function (Pranada *et al.*; 2004). The rationale for using these tags is that they allow two-step purification under denaturing conditions, which is essential to inactivate deubiquitylating enzymes that would otherwise remove polyUb chains and the associated E3 during purification (Tagwerker *et al.*; 2006). A recombinant AV would be generated to ensure efficient expression of the TAP-tagged STAT3 in HUVECs to the same level as endogenous STAT3. After confirming functional expression, it

would be co-expressed with the $A_{2A}AR$ and purified by sequential Ni^{2+} chelate- and streptavidin-affinity chromatography steps following treatment with cytokine and MG132 to promote accumulation of ubiquitylated STATs. To prevent dissociation recruited E3s following denaturing cell lysis, cellular protein would be cross-linked by treatment with formaldehyde prior to lysis. STAT-associated proteins from the final streptavidin-affinity step would be eluted and digested with trypsin for analysis of the resulting peptides by tryptic peptide mass fingerprinting using matrix-assisted laser desorption/ionisation-time of flight (MALDI/TOF) (Bito *et al.*; 2003) and/or peptide separation by nano-liquid chromatography and analysis by tandem mass spectrometry (LC-MS/MS) (Xu and Peng; 2006). Result would be used to interrogate EBI and NCBI human databases using publicly available MASCOT search algorithms. GFP-expressing HUVECs would be used as a negative control.

B) E3 Ub ligase siRNA library screening

Libraries of validated siRNAs specific for more than 200 human E3 Ub ligases are now available commercially from Dharmacon and Ambion. These provide a rapid means with which to screen a range of structurally diverse E3s based on whether knockdown blocks sIL-6Rα/IL-6-mediated degradation of STATs in $A_{2A}AR$ overexpressing HUVECs compared with non-targeting siRNA-treated control cells. This approach is particularly suited to HUVECs since our lab (Sands *et al.*; 2006) and others (Huang *et al.*; 2005; Kooistra *et al.*; 2005) have been able to achieve almost complete target gene knockdown following siRNA transfection into these cells. We would initially start by screening large numbers of group by dividing the library into manageable groups of 50 then screen them to identify the effective group. After that we could further subdivided any putative group over several rounds of screening to isolate the E3 ligase activity which responsible for STATs ubiquitination. To confirm that it functions as a bona fide Ub E3 ligase on phospho-STAT3, those that are identified from our screen will be expressed, purified and used *in vitro* Ub E3 ligase assays using phospho-STAT3 as a substrate.

3) Identification of Ub-conjugation sites and nature of chain linkage on STAT3

Identifying the sites of Ub attachment would define new targets on STAT amenable to therapeutic manipulation. However identification is hampered by the lack of any consensus ubiquitylation sequences displayed by E3s (Nalepa *et al.*; 2006) and (assuming the ubiquitylation sites are conserved) the presence of more than 20 common Lys between STATs 1 and 3. Thus, Ub attachment sites should ideally be determined directly. To achieve this, adenoviruses (AV) would be used to co-express Flag-Tagged-WT STAT3 with the $A_{2A}AR$ prior to treatment with cytokine and proteasome inhibitor MG132 to promote accumulation of ubiquitylated STAT3 (as described in Chapter 2). Flag-STAT3 could then be affinity purified using anti-flag antibody M2-Sepharose columns and eluted with Flag peptide. Following SDS-PAGE and Colloidal Blue staining, bands corresponding to ubiquitylated Flag-STAT3 would be excised and destained prior to in gel digestion with trypsin and peptide extraction. Conjugated ubiquitins are digested by trypsin to either a Gly-Gly remnant that adds a mass of 114.043 Da to the affected Lys residue on the tagged protein or a longer Leu-Arg-Gly-Gly remnant due to miscleavage (Xu and Peng; 2006). Moreover, the ubiquitinated Lys residue on STAT3 will be resistant to trypsin cleavage. Following peptide separation by nano-liquid chromatography and analysis by tandem mass spectrometry (LC-MS/MS), these modifications are detectable as distinctive mass spectrometry (MS/MS) spectra that can be matched to specific Lys residues on STAT3 using search algorithms (Xu and Peng; 2006). A similar approach has been successfully employed to isolate and identify proteins conjugated to NEDD8, a molecule related to Ub (Norman and Shiekhattar; 2006). Its success depends largely on the ability to sufficient purify enough of ubiquitylated STAT3 for mass spectrometry.

To determine the nature of Ub chain linkage, HA-tagged Ub molecules in which all Lys residues (Ub contains seven Lys residues Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) could be individually mutated to Arg, co-expressed with the

A_{2A}AR and tested for their ability to form Ub chains by probing anti-STAT3 immunoprecipitates with anti-HA antibody. Attenuated incorporation of mutated Ub where different Lys residues are mutated to Arg versus WT would identify the Lys residues involved in chain formation. A similar approach, using a mutated Ub in which all seven Lys residues are mutated to Arg, would help determine whether STAT3 is polyubiquitylated or multimonoubiquitylated. This is because the mutated Ub cannot support chain elongation.

4) **Identification of the A_{2A}AR-activated signalling pathway(s) responsible for priming STAT degradation**

Having identified the molecular events by which STAT degradation occurs, it would be important to identify which A_{2A}AR-activated signalling pathways are responsible. A_{2A}AR activation has been reported to activate at least two primary signalling cascades in vascular endothelial cells. One leads to Adenylyl cyclase activation which results in the elevation of intracellular cyclic AMP (cAMP) levels, leading to the activation of cAMP-dependent protein kinase (PKA) and exchange protein activated by cAMP (Epac), which functions as a guanine nucleotide exchange factor for the Rap family of small G-proteins (Tasken and Aandahl; 2004). Another pathway is the Extracellular signal-regulated kinase (ERK). cAMP-independent activation of ERK by the A_{2A}AR (Sexl *et al.*; 1997) is thought to be required for receptor-mediated generation of nitric oxide, which subsequently activates soluble guanylyl cyclase (Wyatt *et al.*; 2002).The resulting accumulation of cyclic GMP (cGMP) activates cGMP-dependent protein kinase.

A range of selective inhibitors and activitors of relevant interacellular signalling pathways will need to be tested for their ability to either block or mimic A_{2A}AR-overexpression-mediated priming of STAT degradation. Fore example, a contribution of Epac can be tested by determining the extent to which Epac-selective activitior 8-pCPT-2′-O-Me-cAMP (Kooistra *et al.*; 2005) can prime STATs for cytokine-

triggered degradation, and whether its depletion by siRNA abolishes the A_{2A}AR's effect. Our Lab has successful applied such approaches to identify a previously unkown Epac1-regulated anti-inflammatory signalling pathway in vascular Ecs (Sands *et al.*; 2006). To determine whether cAMP or ERK pathways may stimulate by the A_{2A}AR involve in STATs degradation in A_{2A}AR-overexpression cells, a broader range of inhibitors and activators for cAMP and ERK will initially be screened for their ability to block STAT degradation prior to performing more discriminating siRNA-mediated knockdown experiments to assess any potential role of candidate intermediate proteins downstream of the A_{2A}AR. Such as the effect of PKA could be inhibited by PKA inhibitors Rp-8-CPT-cAMPS (Park *et al.*; 2007) or H-89 (Kaneto *et al.*; 2007). In addition, we could activate PKA by PKA-specific activator N(6)-benzoyladenosine 3',5-cyclic monophosphate (N(6)Bz-cAMP) (Kwan *et al.*; 2007) or by the adenylyl cyclase activator forskolin (FSK) (Keller *et al.*; 2007). On the other hand, the involvement of ERK in STAT degradation in A_{2A}AR-overexpressing HUVECs could be test by using MEK inhibitor (U0126) (Ciccarelli *et al.*; 2005). The effect of Raf /MEK/ERK pathway activation could be investigated by expressing a Raf-ER fusion protein in A_{2A}AR-overexpressing cells and activating the ER by selective ER activator 4-hydroxytamoxifen. This will specifically activate the ERK pathway and will provide some insight its role as a possible STAT degradation signal in A_{2A}AR overexpressing cells.

Chapter 8

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